Microbiology

Laboratory Manual

BS Part-III

Microbial Anatomy

And

Physiology

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BIOSAFETY RULES

1. Do not eat or drink in the laboratory, chew gum, or place any object on or near your mouth.

All books, backpacks, coats, purses, etc. should be cleared from your table before beginning any procedure. While actively working with microbial cultures, keep your laboratory manual / instructions at a reasonable distance from your work area. Do not wear loose articles of clothing, such as scarves or bulky jackets.

2. Practice good aseptic technique by performing the following at the **beginning** each class:

a) Tie back long hair.

b) Wear closed footwear to protect the feet.

c) Wear lab coats (preferred) or aprons to protect clothes.

d) Clean lab table with an antiseptic wash.

e) Wash hands thoroughly before starting any lab exercise.

f) Wear disposable gloves when handling pathogenic organisms.

g) Wear safety goggles when conducting procedures that pose a splash or aerosol risk, and when dealing with certain chemicals, such as staining reagents.

3. All cultures, whether test tubes or Petri plates, should be labeled with your name or initials, the date, and the name of the organism. Plates can be written on directly; tape labels should be used for test tubes. Permanent markers designated **only** for laboratory use must be used to label cultures.

4. Most microbial cultures will be inoculated using a sterile loop or needle; once the transfer is complete the loop or needle should be sterilized again. Liquid cultures should be transferred using a sterile pipette and mechanical pipetting apparatus. **No mouth pipetting**!

5. All test tubes should be placed in a rack before transporting them from one area of the lab to another. All lids on all test tubes must be secure.

6. All Petri plates should be taped on both sides before carrying to the incubators.

7. All disposable microbiological waste should be discarded in the BIOHAZARD bag. Used test tubes (labels removed) should be placed in the designated pan to be autoclaved and cleaned.

Used pipettes should be placed immediately into the provided waste container. **Do not place any hazardous or infectious materials in the regular trash. Do not place any hazardous or infectious materials in the sink.**

8. All used glass slides and coverslips should be placed in SHARPS boxes. **Do not discard slides in the biohazard bags.**

9. Practice good aseptic technique by performing the following at the **end** of each lab exercise:

a) Remove gloves **inside out** and place them in the biohazard bag

b) Remove lab coats or aprons, fold them inside out, and place them in the assigned drawer.

c) Remove goggles (if used) and clean with alcohol wipes before placing in cabinet for sterilization

d) Decontaminate your work bench by applying an antiseptic wash

e) Wash your hands thoroughly

10. Practice good aseptic technique when making notes or recording results from your experiments in your lab manual -- Do not write in your lab manual or notebook until AFTER you have **removed** your gloves and apron and **washed your hands**!

11. If an accident should occur, such as a spill or a broken test tube:

a) Inform your instructor immediately

b) Soak the area with disinfectant and cover with paper towels

c) Dispose of contaminated paper towels in the biohazard bag

d) Dispose of contaminated broken glass in the SHARPS container

e) Wash hands thoroughly

12. If an accident results in bacterial culture coming in contact with eyes, mucous membranes or an open wound:

a) Eyes should be flushed immediately at the eye wash station

b) Affected wound should be washed thoroughly with soap and water

c) The student should contact their personal physician for advice on further treatment

13. Accident reports must be filed with the Biosafety committee no more than 48 hours following the accident.

14. Students who are having a medical condition which could compromise their immune system must have written permission from their attending physician in order to participate in microbiology lab.

ASEPTIC TECHNIQUE

1. Disinfect the tables with antibacterial cleaner.

2. Wear gloves and lab aprons.

3. When labeling Petri plates, always write on the bottom of the plate.

4. When using incinerators, give them ample time to warm up before sterilizing loops or needles. DO NOT leave loops or needles unattended in the incinerators. They will melt! You will burn your fingers!

5. When inoculating cultures, always sterilize your loop or needle **before** going into a culture and **after** transferring it. Sterilize the loop even if you are going back into the same culture again.

6. Make sure you let your loop cool first – you don't want to kill the bacteria.

7. After removing the lid of a test tube, briefly flames the mouth of the tube before inserting your inoculating loop, and flame again before replacing the cap.

8. Don't put **anything** down on the table – loops, needles, pipettes, test tube lids, etc. Once they touch the table they are **no longer sterile**.

9. Loops/needles can be placed temporarily in slots on the sides of incinerators.

10. Test tube lids can be held with pinky finger.

11. Pipettes should remain in canister until just before use.

12. Don't leave media open to the air – bacteria and fungi in the air can contaminate the media.

13. Don't over-inoculate! It doesn't take much inoculum to start a culture. Simply touch the loop or needle to the bacterial growth and obtain a **small amount** on the loop. Don't "scrape" the culture, and don't dig into the agar.

14. When streaking onto an agar plate or slant, make sure the loop doesn't break the surface of the agar. A gentle gliding motion is all that is necessary to distribute the bacteria on the plate.

15. When finished, disinfect the tables again with antibacterial cleaner. Dispose of gloves in the biohazard trash, and place aprons back in your designated drawers. Wash your hands!

Learning Objectives

- To get familiar with rules and regulation of Microbiology laboratory.
- Adopt safety measures when performing any experiments, particularly those concerned with infectious or pathogenic organisms, in the laboratory.
- To learn the importance of Aseptic techniques for a successful experiment.

Questions OR Lab quiz:

1. What are the aseptic techniques?

2. What is sterilization and why it's necessary to sterile the apparatus before and after the experiments in Microbiology work?

3. Discuss the importance of laboratory safety rules.

List of Important terms and function (s) of basic equipments and tools used in Microbiology Laboratory

CULTURE MEDIUM: The survival and continued growth of microorganisms depends on an adequate supply of nutrients and a favorable growth environment. In order to continue the growth microbes must use soluble low molecular weight substances that are frequently derived from the enzymatic degradation of complex nutrients. A solution containing these nutrients is called **culture medium**. Basically, all culture media are liquid, semisolid or solid.

COLONY: A colony is cluster of cells derived from the multiplication of a single cell and represent the growth of a single species of microorganisms.

PURE CULTURE: A defined or well-isolated colony is a pure culture OR growth of a single species of microorganism.

AGAR SLANTS AND DEEP TEST TUBES: When a solid medium in liquid state is placed in test tubes and are then allowed to solidify in a slanted position, produce an agar slant. These are useful in maintaining pure cultures. When solid medium is allowed to harden in the upright position test tubes, it is designated as agar deep tubes, which are used primarily for the study of the gaseous requirements of microorganisms.

WIRE LOOP AND NEEDLES: They are made from inert metals such as platinum and are inserted into metal shafts that serves as handles. They are extremely durable instruments and are easily sterilized by incinerations in the hottest portion of Bunsen burner flame.

INCUBATOR: An incubator is used to maintain optimum temperature during necessary growth period. It resembles and oven and thermostatically controlled so that temperature can be varied depending on the requirement of specific microorganisms. Most incubators use dry heat. Moisture is supplied by placing a beaker of water in the incubator during

growth period. A moist environment retards dehydration of the medium and thereby avoids false experimental results.

OVEN: Oven provides dry heat and primarily used for the sterilization of petriplates and other glass wares, like test tubes and pipettes. The sterility of glass wares is achieved at the temperature of 160-170 ^{0}C for 1-2 hours.

AUTOCLAVE: Autoclave used primarily for the sterilization of culture medium. It provides moist heat under pressure. Sterility is achieved at 121^{0}C for 15-20 minutes of incubation.

REFRIGERATOR: A referigerator is used for a wide variety of purposes such as maintenance and storage of stock cultures between subculturing period and storage of sterile media to prevent dehydration. It is also used as a repository for thermostable solutions, antibiotics, serums, and biochemical reagents.

CULTURE MEDIUM

Microscopic examination of microorganisms provides important information about their morphology but does not tell us much about their biological characteristics. To obtain such information, we need to observe microorganisms in **culture.** If we are to cultivate them successfully in the laboratory, we must provide them with suitable nutrients, such as protein components, carbohydrates, minerals, vitamins, and moisture in the right composition. This mixture is called **a culture medium** (plural, **media**). It may be prepared in liquid form, as a **broth,** or solidified with agar. **Agar** is a **nonnutritive** solidifying agent extracted from seaweed, a complex carbohydrate mainly composed of galactose. Agar serves as an excellent solidifying agent because it liquefies at **100^0C** and solidifies at **40^0C**. Because of these properties, organisms, especially pathogens, can be cultivated at temperatures of **37.5^0C** or slightly higher without fear of medium liquefying. A completely solid medium requires an agar concentration of about **1.5** to **1.8**%. A concentration of less than **1%** agar results in a **semisolid medium**.

Agar media may be used in tubes as a solid column (called a **deep**) or as **slants,** which have a greater surface area (see Figure. 2c). They are also commonly used in **petri dishes** (named for the German bacteriologist who designed them), or plates.

Solid media are essential for isolating and separating bacteria growing together in a specimen collected from a patient, for example, urine or sputum. When a mixture of bacteria is streaked (spread) across the surface of an agar plate, it is diluted out so that single bacterial cells are deposited at certain areas on the plate. These single cells multiply at those sites until a visible aggregate called a **colony** is formed (see Figure. 2D). Each colony represents the growth of one bacterial species. A single, separated colony can be transferred to another medium, where it will grow as a **pure culture.**

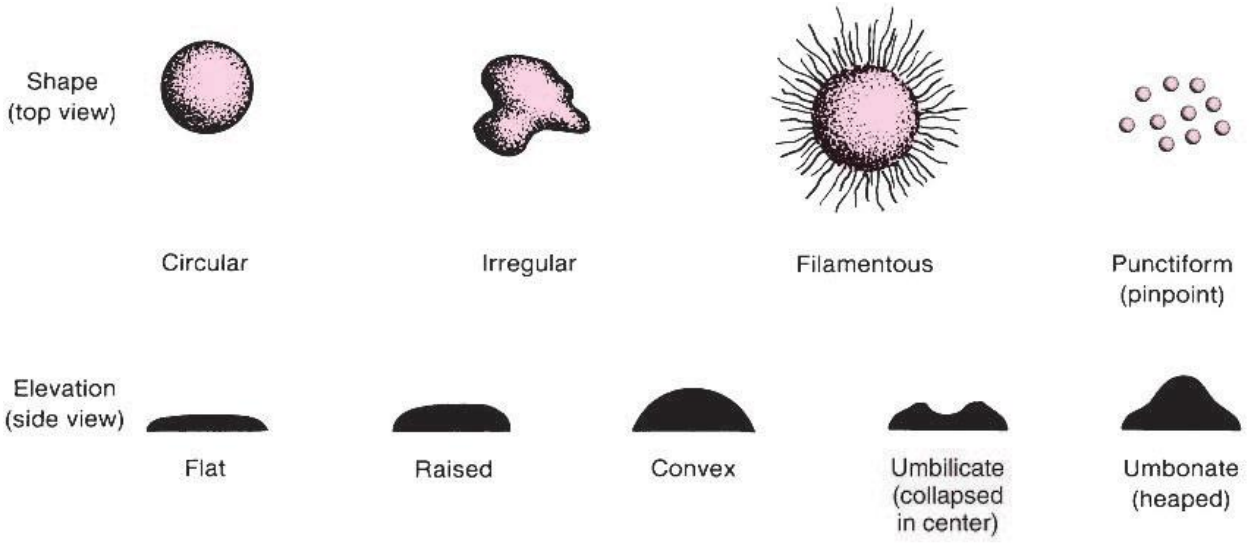
Colonies of several different species are regularly present on the same agar plate when certain patient specimens are inoculated onto them. Work with pure cultures permits the microbiologist to study the properties of individual species without interference from other species. This practice of streaking plates to obtain pure cultures is critical in the hospital laboratory because it allows the microbiologist to determine how many types of bacteria are present, to identify those likely to be causing the patient's disease, and to test which antimicrobial agents will be effective for treatment.

The appearance of colonial growth on agar media can be very distinctive for individual species. Observation of the noticeable, gross features of colonies, that is, of their **colonial morphology**, is therefore very important. The color, density, consistency, surface texture, shape, and size of colonies all should be observed, for these features can provide clues as to the identity of an organism, although final identification cannot be made by morphology alone.

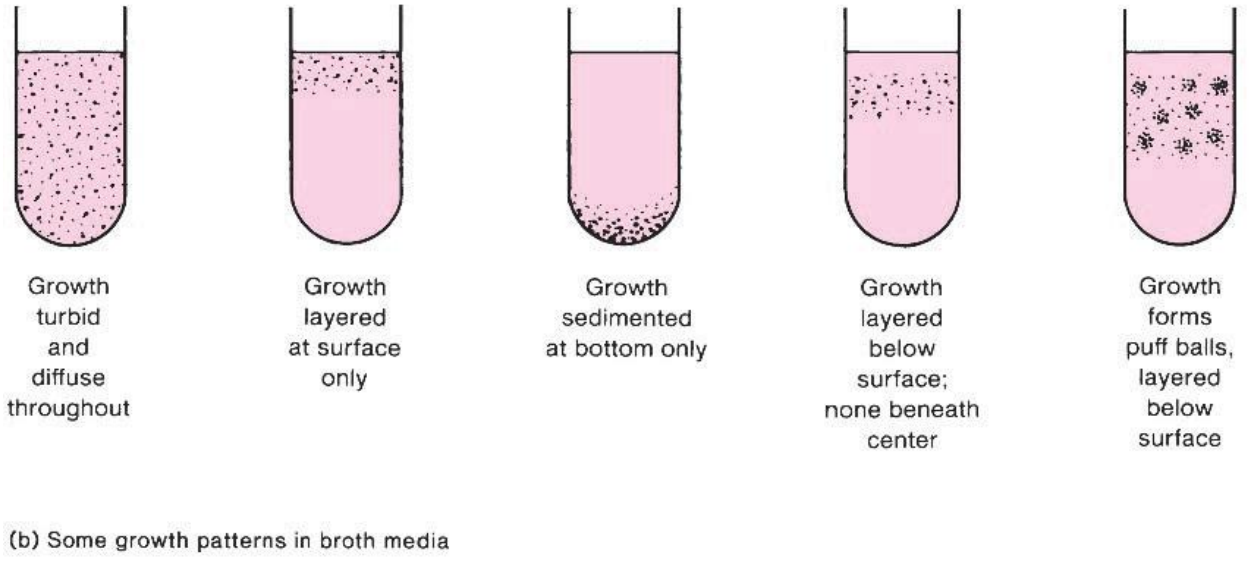
In liquid media, some bacteria grow diffusely, producing uniform clouding, whereas others look very granular. Layering of growth at the top, center, or bottom of a broth tube reveals something of the organisms' oxygen requirements. Sometimes colonial aggregates are formed and the bacterial growth appears as small puff balls floating in the broth. Observation of such features can also be helpful in recognizing types of organisms (see Figure 1).

Note to remember: You must learn how to handle cultures aseptically.

The organisms must not be permitted to contaminate the worker or the environment, and the cultures must not be contaminated with extraneous organisms. Also, if you contaminate the cultures, your results will be spoiled. Before you begin, reread the Section dealing with Biosafety rules.



(a) Some colonial characteristics on agar media*



(b) Some growth patterns in broth media

Figure 1. Examples of bacterial growth patterns. **(a)** Some colonial characteristics on agar media. Characteristics of the colony edges may be distinctive for many bacterial species. The shapes and elevations shown in the two rows of sketches are not intended to be matched.

*Note: Shapes and elevations shown in this diagram are not intended to be matched.

(b) Some growth patterns in broth media.

Lab Quiz:

1. Define the terms highlighted as **bold** in text above.

Preparation of culture medium

Function of culture medium

- **All-purpose media**, such as, **Nutrient agar medium**, do not contain any special additives and they aim to support the growth of most bacteria.
- **Selective media** enhance the growth of certain organisms while inhibit others due to the inclusion of particular substrate(s).
- **Differential media** allow identification of microorganisms usually through their unique (and visible) physiological reactions. In the detection of common pathogens, most practical media are both selective and differential.
- **Enrichment media** contain specific growth factors that allow the growth of metabolically fastidious microorganisms. An enrichment culture is obtained with selected media and incubation conditions to isolate the microorganisms of interest.

PRECAUTIONS

Culture media must be stored at the specified temperature, under specified conditions and not longer than the shelf-life periods appropriate to each product. The storage conditions and expiry date of each product are shown on the labels or product inserts but the following general rules will help to ensure that they are kept in an optimum environment. When storing products note the shelf life expiry dates on the labels and use the products in order of their lot/batch numbers.

Light

All prepared culture media and their components should be stored away from light and exposure to direct sunlight should be avoided at all times.

Humidity

Sealed glass and plastic containers are unaffected by normal laboratory humidity. Opened containers of dehydrated powders will be affected by high humidity. Hot, steamy media preparation rooms are not suitable environments to store containers of culture media; particularly containers which are frequently opened and closed. An adjacent cold room or adequate storage cupboards are preferable storage areas.

Preparation of dehydrated media

Dehydrated media are hygroscopic and are sensitive to moisture, heat and light. They are adversely affected by drastic changes in temperature e.g. hot/cold cycling temperatures which may occur between day and night laboratory temperatures in winter. Storage conditions are usually indicated on the product label and should be followed.

Reconstitution of dehydrated media

Complete instructions for the preparation of culture media are given on the label of each bottle. As a general rule it is wise to prepare one week's requirement only.

1. Always use freshly prepared distilled or deionized water. Use warm (50°C) water to hasten the solution of the medium. Rinse all glassware with the distilled/deionized water and make sure that the vessels are clean and free from toxic chemicals which may be absorbed on to the surface of the glass e.g. bile salts, tellurite, selenite etc.

2. Prepare the medium in a vessel about twice the final volume of the medium to allow adequate mixing. Follow the instructions given on the label of each product.

3. Open the culture medium container away from draughts and moisture. Avoid inhaling the powder and prolonged skin contact. Weigh the powder quickly, accurately and without creating 'clouds of dust'. Reclose the container as soon as possible. Pour

half the required volume of distilled water in the vessel, then the weighed quantity of medium and agitate briskly for a few minutes. Pour the rest of the distilled water down the sides of the vessel to wash any adherent medium back into solution. This is an important step because dry culture media powder above the level of the water may not be sterilized in the autoclave and may be a source of contamination.

Agar-free media will usually dissolve on gentle agitation. Media containing agar should be **heated** to dissolve the agar **before** autoclaving. Bring the medium to the boil without scorching or burning. Most culture media will require final sterilization in an autoclave at **121°C** for **20 minutes**.

The pH of the dehydrated medium has been adjusted by the manufacturer so that the final pH of the prepared medium conforms with the label specification when the medium has been cooled to 25°C. Do not adjust the pH of dehydrated media prior to sterilization.

Sterilization of culture media

Although sterilization of culture media is best carried out in a steam autoclave at temperatures between 121-134°C it has to be recognized that damage is caused to the medium by the heating process. Heat-treatment of complex culture media which contain peptides, sugars, minerals and metals results in nutrient destruction, either by direct thermal degradation or by reaction between the medium components. Toxic products caused by chemooxidation can also be formed during heat-treatment. It is important, therefore, to optimize the heating process so that a medium is sterile after heating but minimal damage is caused to the ingredients of the medium. As a general rule it is accepted that short-duration, high-temperature processes are more lethal to organisms and less chemically damaging than are longer, lower temperature processes e.g. 3 minutes at 134°C is preferable to 20 minutes at 115°C.

A general instruction for sterilizing culture media in volumes up to one liter at 121°C for 20 minutes is given on each label.

Overheating effects

Overheating is a common cause of pH drift, darkening, precipitation, poor gel strength and reduced bacteriological performance. These effects can also be produced if a concentrated 'pool' of ingredients at the bottom of the container is heated. **All culture media should be in solution before sterilization**. This will reduce the occurrence of Maillard-type reactions (non-enzymatic browning) taking place in the medium.

EXPERIMENT 1- PREPARATION OF AGAR PLATES

Materials and equipment

Distilled water, measuring cylinder, flask, bacteriological media, laboratory scales, chemical spoons, cotton gloves, sterile, empty Petri dishes, Bunsen burner, autoclave, incubator. **Note**: pH indicator paper or pH meter, 1N NaOH solution, 1N HCl solution are required If pH adjustment is necessary.

1. Measure the components of the medium into a flask. Use a clean chemical spoon for every measurement. Dissolve the solid components and fill with the solvent up to final volume.
2. Close the flask with cotton plug and cover with aluminum foil, put into the autoclave for sterilization. When the internal temperature reached 121°C, every component (e.g. agar-agar) will be dissolved correctly, at that temperature.
3. Cool the sterilized medium to 55°C.
4. Take out the cotton plug and flame the mouth of the flask over a Bunsen burner, and then pour the medium into sterile, empty Petri dishes (15-20 mL into each Petri dish).
5. Keep the Petri dishes horizontally until the medium completely solidifies. Turn dishes upside-down and stack them up for storage.
6. Label the plates according to the type of the medium and perform a sterility test: Incubate the test tubes at 28-35°C for 24 hours, and check for sterility.
7. The prepared media can be stored for 1-2 weeks at 12-15°C, or longer in a refrigerator. (Do not store medium containing agar-agar under 4-5°C as it destroys its structure!). In case of longer storage, Petri plates must be placed into plastic bags or boxes to avoid drying out.

EXPERIMENT 2- CULTURE TRANSFER TECHNIQUES

The purpose of this lab exercise is to learn how to subculture microorganisms in various types of microbiological media. Simply stated, **subculturing** is transferring microorganisms from one media type to another. Various media types used in microbiology labs include agar slants, agar deeps, agar plates, and broths. An **agar slant** is a solid media in a test tube with a slanted surface on which to culture the microorganism. These are typically inoculated by streaking the surface of the slant with a sterile loop. An **agar deep** is a solid media in a test tube which does not have a slanted surface. These are typically inoculated by stabbing the media with a sterile needle. An **agar plate** is a solid media which is contained in a Petri plate, providing an optimal surface on which to culture microorganisms. Like the agar slants, these are inoculated by streaking the surface with a sterile loop. **Broth tubes** are a liquid medium which can be inoculated by a sterile loop, needle, or pipette.

Remember that when inoculating any cultures, it is essential to practice sterile technique at all times! It is important to know what **sterile media** looks like. Take a minute to observe your **uninoculated** cultures. You will be responsible for making sure there are no contaminates present in the media we provide for you.

MEDIA NEEDED:

1 Nutrient agar slant

1 Nutrient agar deep

1 Nutrient broth tube

CULTURES NEEDED: *Depending on any bacterial culture availability.*

PROCEDURE:

1. **Label** each tube appropriately.

2. Using a sterile **needle**, obtain a small amount of culture from the **broth** tube containing bacterial culture, for example, *E.coli.* Use the needle to inoculate a nutrient agar deep tube by stabbing the needle into the agar deep.

3. Using a sterile **loop**, obtain a loop full of culture from the **broth** tube containing *E. coli.* Use the loop to inoculate a nutrient agar slant tube by streaking the agar slant with a zig-zag motion.

4. Using a sterile **loop**, obtain a loop full of culture from the **slant** of *E.coli*. Use the loop to inoculate a nutrient broth tube by gently swishing the loop around in the liquid broth.

5. Make sure all caps are loose, but secure.

6. Incubate at 30-35°C for 24 hours.

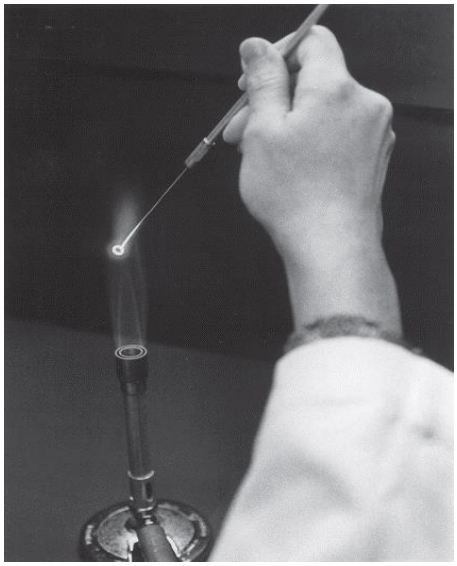
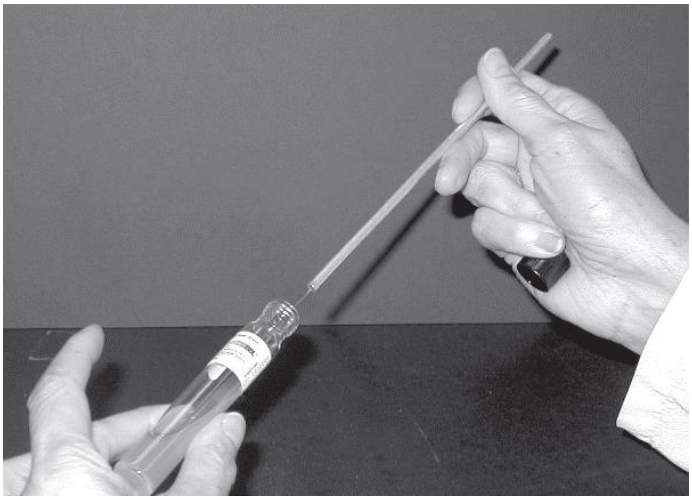
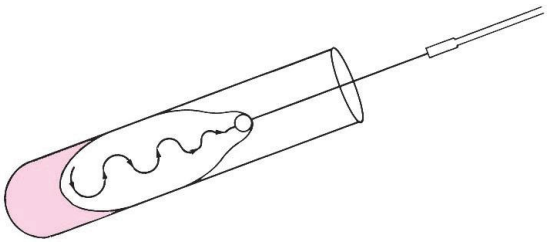


Figure 2. Culture Transfer Techniques.

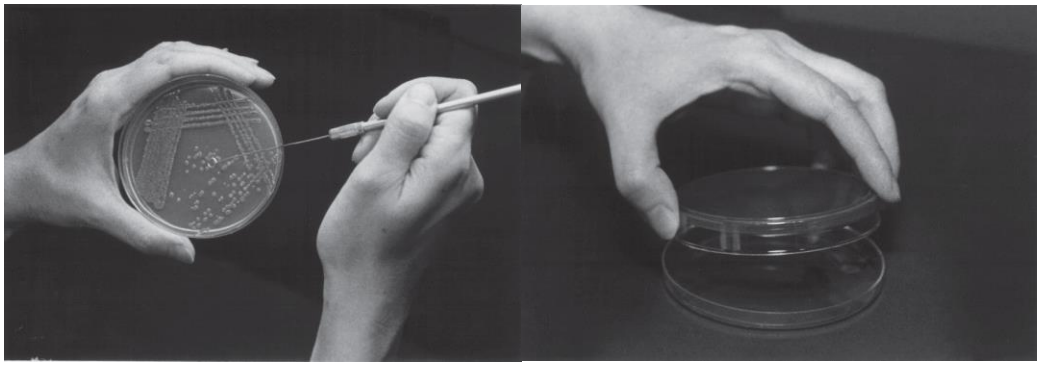
A. Sterilizing the wire inoculating loop in the flame of a Bunsen burner



B. Inoculating a culture tube. Notice that the tube is held almost horizontally. Its cap is tucked in the little finger of the right hand, which holds the inoculating loop.



C. Streaking an agar slant with the loop.



D. Opening a petri plate culture. The bottom is lifted out of the top, and the top is left lying face up on the bench (Right side).

Selecting an isolated bacterial colony from a plate culture surface. The plate has been streaked so that single colonies have grown in well-separated positions and can easily be picked up.

RESULTS:

Examine broth tubes, agar deep tubes and agar slant tubes. Make and complete the chart below:

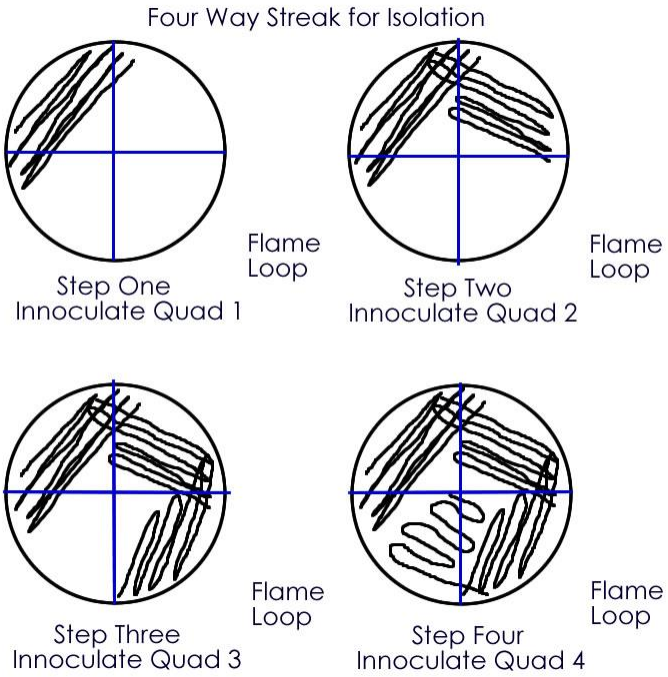
	Nutrient Broth	Nutrient Agar Slant	Nutrient Agar plate
Growth (+) or (-)			
Pigmentation (+) or (-)			
Sketch the distribution of growth			

STUDY QUESTIONS

1. Why is it important to sterilize the inoculating instrument before and after each inoculation?
2. Why did we use an inoculating needle to inoculate the agar deep tube?
3. Why can a single colony on a plate be used to start a pure culture?
4. How would you determine whether culture media given to you are sterile before you use them?

EXPERIMENT 3 – ISOLATION OF PURE CULTURES

The purpose of this experiment is to separate a mixed culture of *staphylococcus aureus* and *Escherichia coli* and obtain single, isolated colonies of each bacterial species. When working with microorganisms, it is desirable to start with single, isolated colonies to ensure you are working with a pure culture. Cultures that are visible on the surface of solid media are called colonies. A **colony** forms on a plate when a single microbe is inoculated onto the surface of the plate and reproduces until there are enough cells to form a visible colony. Since a colony theoretically forms from a single cell, a colony should then represent a pure culture. One way to obtain single, isolated colonies is using the quadrant streak method. The quadrant streak plate method allows sequential dilution of the original microbial material over the entire surface of a fresh plate. As the original sample is diluted by streaking it over successive quadrants, the number of organism decreases. Usually by the third or fourth quadrant only a few organisms are transferred, and these produce single, discrete colonies.



MEDIA: *(per student):* Nutrient agar plate

CULTURES: Mixed broth culture of *staphylococcus aureus* and *Escherichia coli.*

PROCEDURE:

1. Divide the agar plate into 4 quadrants.

2. Place a loopful of culture onto the plate in Quadrant 1 with a sterile loop and streak the loop very gently using a back and forth motion.

3. Sterilize loop. Go back to the edge of Quadrant 1 and extend the streaks into Quadrant 2, going back into Quadrant 1 twice.

4. Sterilize loop. Go back to the edge of Quadrant 2 and extend the streaks into Quadrant 3, going back into Quadrant 2 twice.

5. Sterilize loop. Go back to the edge of Quadrant 3 and extend the streaks into Quadrant 4, going back into Quadrant 3 twice. *Be careful NOT to go back into Quadrant 1!*

6. Tape plate closed on both sides. Make sure the plate is labeled with your name, date, and the organism(s), and incubate upside down (to prevent condensation from getting on to agar) at 30-35 °C.

RESULTS

Examine your isolation streak plate. Did you obtain single, isolated colonies?

Do you see two distinct colony types on the agar plate?

Draw the distribution of the growth and colonies on the plate.

STUDY QUESTIONS

1. Why is it important to start a bacterial culture with a single, isolated colony?

2. Suppose you suspect that a supposedly pure bacterial culture is contaminated with another bacterium. Could you use this technique to determine if a contaminant is present? Explain.

EXPERIMENT 4- VIABLE PLATE COUNTS

The purpose of this exercise is to quantify the number of bacteria in a broth culture of *E. coli.* In microbiological research, it is often necessary to be able to **quantify** the number of living bacteria in a particular sample. One of the major ways to do this is using **viable plate counts**, in which bacterial cells from a liquid culture are spread onto an agar plate. The plate is incubated, the numbers of colonies that grow on the plate are counted, and the number of original bacterial cells in the culture is determined. In most cases, however, the liquid culture being quantified contains too many cells to be directly plated onto agar plates – there would be so much growth that it would be impossible to count individual colonies! Therefore, the liquid culture needs to be diluted, often 1-million-fold, before it can be plated. When such a large dilution is required, an accurate dilution cannot be made in a single dilution step and it is necessary to make **serial dilutions**. Serial dilutions are a step-wise set of dilutions which sequentially dilute the bacterial culture. One or more of the dilutions are then plated on the agar plates to determine the number of colonies present in the original culture. Only plates containing between 30 and 300 colonies are counted to ensure statistically significant data. To estimate the number of bacterial in the original culture, the number of colonies on the plate is multiplied by the total dilution plated. For example, suppose 0.1 ml of a 10^6 dilution was plated, and 123 colonies were counted following incubation. The **total dilution plated** would be 10^7 (since only 0.1 ml was plated), and the number of bacteria/ml of the original culture would be: (123) x $1/10^7$ = 1.23 x 10^9 CFU/ml. Note that the results are expressed as “colony forming units (CFU)” per ml.

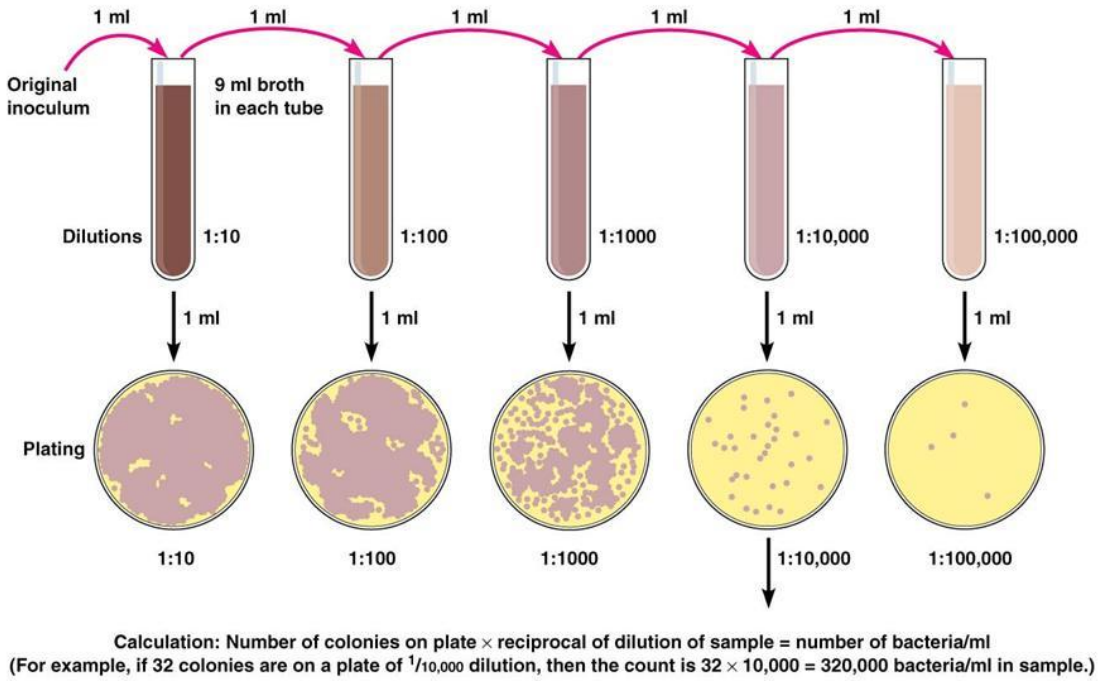


Figure 4. Viable plate count method.

MEDIA NEEDED *(per group of four)*

3 Nutrient agar plates, 7 Dilution blanks containing 9 ml water

CULTURES NEEDED

Overnight broth culture of *Escherichia coli*

PROCEDURE

1. Label the dilution blanks as follows: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}.

2. Label the agar plates as follows: 10^{-6}, 10^{-7}, 10^{8}.

3. Using a sterile pipette, transfer 1 ml of the *E. coli* broth culture into the tube labeled 10^{-1}. Mix thoroughly.

4. Using a new sterile pipette, transfer 1 ml of the 10^{-1} tube into the tube labeled 10^{-2}. Mix thoroughly.

5. Using a new sterile pipette, transfer 1 ml of the 10^{-2} tube into the tube labeled 10^{-3}. Mix thoroughly.

6. Using a new sterile pipette, transfer 1 ml of the 10^{-3} tube into the tube labeled 10^{-4}. Mix thoroughly.

7. Using a new sterile pipette, transfer 1 ml of the 10^{-4} tube into the tube labeled 10^{-5}. Mix thoroughly.

8. Using a new sterile pipette, transfer 1 ml of the 10^{-5} tube into the tube labeled 10^{-6}. Mix thoroughly.

9. Using a new sterile pipette, transfer 1 ml of the 10^{-6} tube into the tube labeled 10^{-7}. Mix thoroughly.

10. Using a new sterile pipette, transfer 0.1 ml of the 10^{-5} tube to the nutrient agar plate labeled 10^{-6}, and spread the liquid thoroughly and evenly over the surface of the plate using a sterile disposable spreader. Be careful not to let the spreader dig into the agar! [Note: Since we're only plating 0.1 ml of the 10^{-5} dilution, the total dilution plated is 10^{-6}.]

11. Using a new sterile pipette, transfer 0.1 ml of the 10^{-6} tube to the nutrient agar plate labeled 10^{-7}, and spread the liquid thoroughly and evenly over the surface of the plate using a sterile disposable spreader.

12. Using a new sterile pipette, transfer 0.1 ml of the 10^{-7} tube to the nutrient agar plate labeled 10^{-8}, and spread the liquid thoroughly and evenly over the surface of the plate using a sterile disposable spreader.

13. After the liquid has absorbed into the plates, tape them closed on both sides. Make sure your plates are labeled with your name, date, and the organism, and incubate upside down at 30-35 °C.

RESULTS

Examine the nutrient agar plates for growth, and count the number of colonies on each plate. Remember, the number has to be between 30 and 300 in order to be statistically accurate. If your plate has fewer than 30 colonies, record the number as “TFTC” for “too few to count”. If your plate has more than 300 colonies, record the number as “TNTC” for “too numerous to count”. Then, use the formula on the previous page to determine the number of CFU/ml of the original broth culture.

Dilution	Number of colonies	Number of CFU/ml of original broth culture *(Remember to use scientific notation!)*
10^{-6}		
10^{-7}		
10^{-8}		

STUDY QUESTIONS:

1. Why do you think it is important to be able to quantify the number of viable bacteria in a sample?
2. Define CFU. How would you calculate CFU/ml?

EXPERIMENT 5- STREAKING TECHNIQUE TO OBTAIN PURE CULTURES

Purpose: To isolate pure cultures from a specimen containing mixed flora.

The skin and many mucosal surfaces of the human body support large numbers of microorganisms that comprise the normal, or indigenous, flora. When clinical specimens are collected from these surfaces and cultured, any pathogenic microorganisms being sought must be recognized among, and isolated from, other harmless organisms. Colonies of the pathogenic species must be picked out of the *mixed* culture and grown in isolated *pure* culture. The microbiologist can then proceed to identify the isolated organism by examining its biochemical and immunological properties. Pure culture technique is critical to successful, accurate identification of microorganisms.

Materials

Nutrient agar plates, Sterile swabs

A mixed broth culture containing *Serratia marcescens* (pigmented), *Escherichia coli,* and *Staphylococcus epidermidis*

A demonstration plate culture made from this broth, showing colonies isolated by good streaking technique

Glass slides, Gram-stain reagents

PROCEDURE

Streaking a Mixed Broth Culture for Colony Isolation

1. Make certain the contents of the broth culture tube are evenly mixed.
2. Place a loop full of broth culture on the surface of a nutrient agar plate, near but not touching the edge. With the loop flat against the agar surface, lightly streak the inoculum back and forth over approximately one-eighth the area of the plate; do not dig up the agar (Figure 5, area A).
3. Sterilize the loop and let it cool in air.

4. Rotate the open plate in your left hand so that you can streak a series of four lines back and forth, each passing through the inoculum and extending across one side of the plate (Fig. 5, area B).

5. Sterilize the loop again and let it cool in air.

6. Rotate the plate and streak another series of four lines, each crossing the end of the last four streaks and extending across the adjacent side of the plate (Fig. 5, area C).

7. Rotate the plate and repeat this parallel streaking once more (Fig.5. area D).

8. Finally, make a few streaks in the untouched center of the plate (Fig. 5, area E). *Do not touch the original inoculum.*

9. Incubate the plate (inverted) at 35°C.

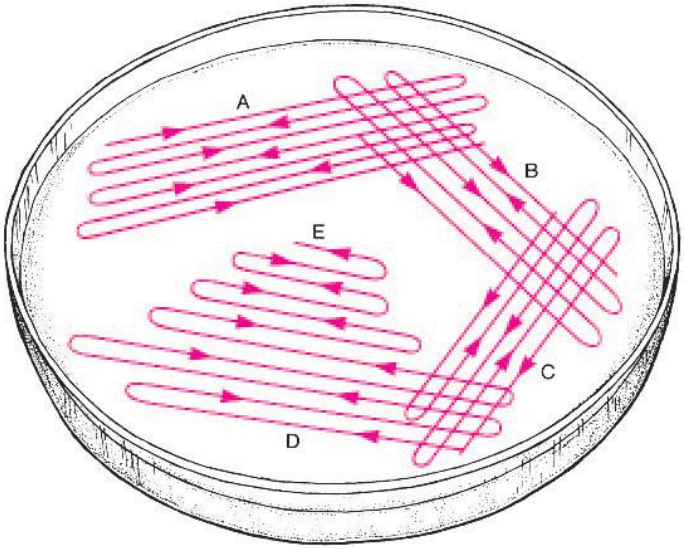


Figure 5 Diagram of plate streaking technique. The goal is to thin the numbers of bacteria growing in each successive area of the plate as it is rotated and streaked so that isolated colonies will appear in sections D and E.

RESULTS

Examination of Plate Streaked from Mixed Broth Culture

1. Examine the incubated nutrient agar plate carefully. Compare your streaking with that illustrated in figure 8.1a and b.

2. Make a drawing of your result showing the intensity of growth in each streaked area as it is presented in Figure 8.1.

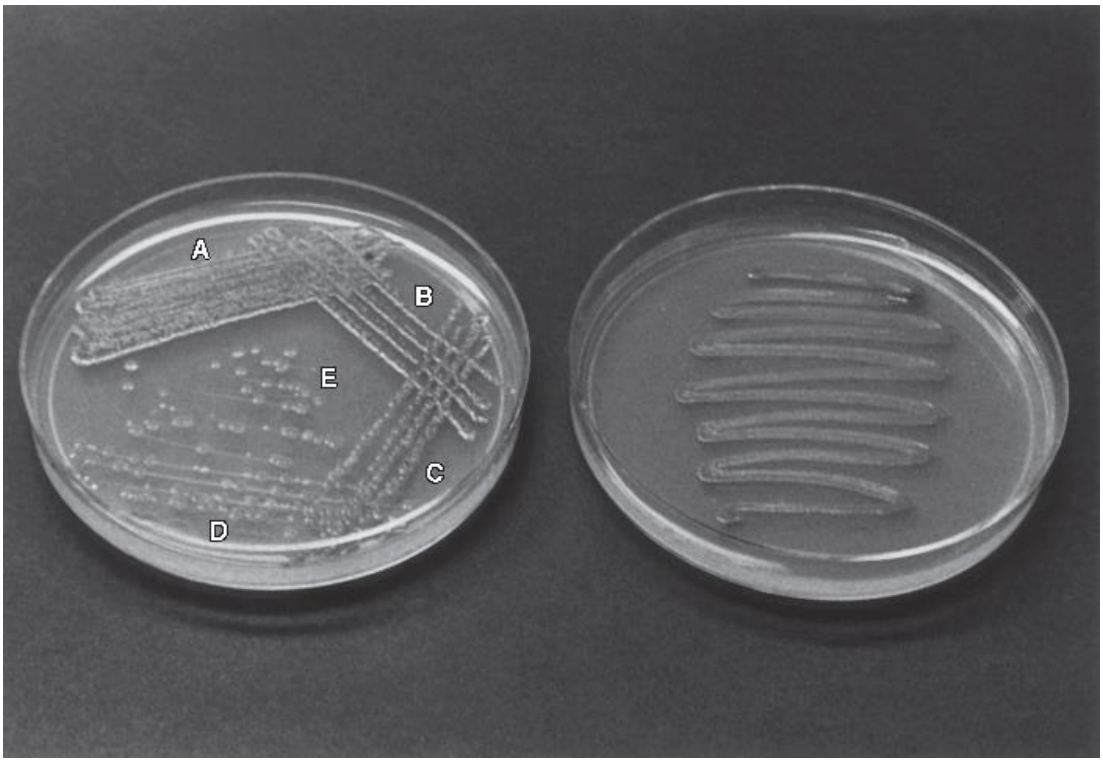


Figure 5.1 Plate streaking. (a) Notice how the proper technique is designed to yield isolated colonies in areas D and E. (b) Poor streaking does not provide separation of colonies.

3. Make a Gram stain of one isolated colony of each type present. Also prepare a Gram stain of the growth in the area where the initial inoculum was placed. (Note: when a stain is to be made of colonies on an agar medium, place a loop full of sterile water or saline on the slide first and then emulsify the picked growth in this drop. Allow to air dry, fix the slide by heat or methanol, and stain.)

4. Record your observations in the table provided.

Study Questions

1. When an agar plate is inoculated, why is the loop sterilized after the initial inoculum is put on?
2. Define a pure culture and mixed culture.
3. Define a bacterial colony. List four characteristics by which bacterial colonies may be distinguished.
4. Why should a petri dish not be left open for any extended period?
5. Why does the streaking method you used to inoculate your plates result in isolated colonies?

EXPERIMENT 6- POUR-PLATE TECHNIQUE

PURPOSE: To learn the pour-plate technique for obtaining isolated colonies**.**

An alternative method for using agar plates to obtain isolated colonies, other than streaking their surfaces, is to prepare a “pour plate.” In this case, an aliquot of the specimen to be cultured is placed in the bottom of an empty, sterile petri dish, then melted, cooled agar is poured over it. Quickly, before the agar cools, the plate is gently rocked to disperse the inoculum. When the agar has solidified and the plate is incubated, any bacteria present in the specimen will grow wherever they have been embedded within the agar layer or localized on its surface. Their colonies will be isolated and can be removed from subsurface positions by inserting the inoculating loop or a straight wire into the agar. To prepare pour plates, the inoculum must be a liquid specimen or culture. If it is not, it must be suspended in sterile fluid before being placed in the petri dish. Another method for preparing a pour plate is to inoculate the specimen or culture directly into the tube of melted, cooled, but not yet solidified agar. Mix it by rolling it back and forth between the outstretched fingers of both hands, and pour the inoculated agar into a sterile petri dish. These steps must be performed quickly before the agar cools enough to harden. When primary isolation plates have been properly poured or streaked, individual colonies can be picked up on an inoculating loop or straight wire and inoculated to fresh agar or broth media. These new pure cultures of isolated organisms are called *subcultures.*

Materials: Tubed nutrient agar (10 ml per tube)

Sterile petri dishes

Sterile 1-ml pipettes

Mixed broth culture containing any two bacterial species like *Escherichia coli* and *Staphylococcus epidermidis*

Nutrient agar plates, Nutrient agar broth, Water bath.

PROCEDURE: **Pour-Plate Technique**

1. Place a tube of sterile nutrient agar in a boiling water bath. (A simple water bath can be set up by placing a glass beaker or tin can half filled with water on a tripod over a Bunsen flame. An asbestos mat must be used under glass vessels. The water should be kept at a steady but not rapid boil. Keep the water level at the halfway mark. An electric burner may be used instead.)

2. When the agar is liquefied, remove the tube and allow it to cool to about 50°C.

3. Place an empty sterile petri dish before you, top side up.

4. Remove a sterile 1-ml pipette from its container, keeping your fingers on the plugged mouth end pipetting. The pressure of the finger on the mouth of the pipette controls the flow and measurement of the fluid. Notice that the cap from the open tube is held by the little finger of the right hand.

5. Pick up the mixed broth culture in the other hand, remove its closure with the little finger of the hand holding the pipette (*do not touch the pipette to anything*), and insert the pipette into the broth.

6. Holding the tube and pipette vertically, poise your index finger over the pipette mouth. Allow the pipette to fill to the level of the broth in the tube and then close off its mouth with your finger (there should be about 0.3 to 0.4 ml of culture in the pipette).

7. Keeping your finger pressed on its top, raise the pipette until the tip is free of the broth and then slowly allow the material in the pipette to run back into the tube until only the

Figure 6 Pipetting. The pressure of the finger on the mouth of the pipette controls the flow and measurement of the fluid. Notice that the cap from the open tube is held by the little finger of the right hand.

last 0.1 ml remains. Now press your finger tightly to close the pipette's mouth and prevent further dripping (Figure 6). **Never use your mouth to draw fluid into a pipette.**

8. Before you withdraw the pipette from the tube, touch its tip against the dry inner wall to remove any drop hanging from it.

9. Withdraw the closed pipette, replace the tube closure, and put the tube down in the rack.

10. Now, with your free hand, remove the top of the petri dish (do not put it down), place the tip of the pipette against the bottom of the dish, release your finger from the mouth, and let 0.1 ml of broth culture run into the plate bottom.

11. Replace the dish top and discard the pipette into a container of disinfectant.

12. Pick up the tube of melted, cooled agar, remove its closure, and put it down on the bench top.

13. With your free hand, remove the top of the petri dish (again, do not put it down). Quickly pour the agar into the dish.

14. Replace the petri dish cover (the tube may be set aside for washing). Gently rock the closed dish, or rotate it in circular fashion on the bench top, being careful not to allow the still melted agar to wave up over the edge of the bottom half or onto the cover.

15. Let the agar solidify without further disturbance. When it is quite firm (about 30 minutes), invert the plate and place it in the 35°C incubator.

RESULTS

A. Examination of Pour Plate

Diagram the distribution of colonies you can see in your pour-plate culture (surface and subsurface locations, separation). Indicate any colonial distinctions you can recognize. Examine your nutrient broth subcultures. Make Gram stains to determine whether they are pure. Describe your microscopic observations of each broth subculture.

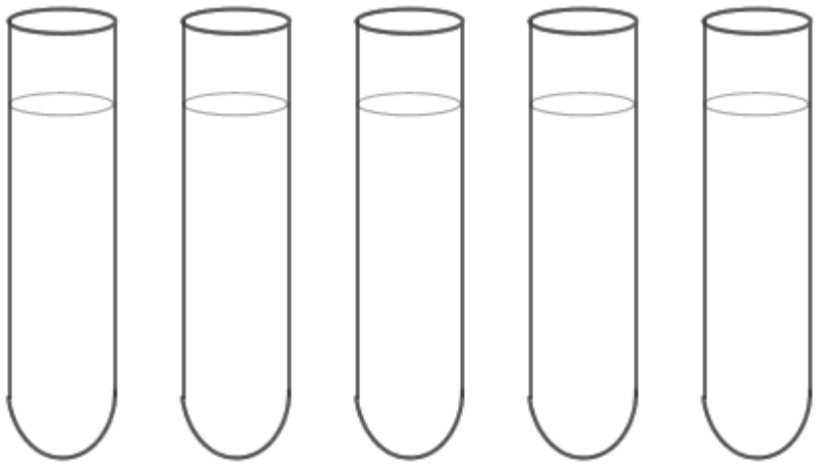
STUDY QUESTIONS

1. Discuss the relative convenience of pour- and streak-plate techniques in culturing clinical specimens.
2. Why are plate cultures incubated in the inverted position?
3. How do you decide which colonies should be picked from a plate culture of a mixed flora?
4. Why is it necessary to make pure subcultures of organisms grown from clinical specimens?
5. How can you determine whether a culture or subculture is pure?
6. When more than one colony type appears in a pure culture, what are the most likely sources of the extraneous organisms?

EXPERIMENT 7- ATMOSPHERIC OXYGEN REQUIREMENT

Bacterial growth is also dependent on the presence of oxygen in the environment, as different bacteria have different oxygen requirements depending on the types of enzymes they possess. The major bacterial oxygen classes are aerobes, microaerophiles, obligate anaerobes, aerotolerant anaerobes, and facultative anaerobes. **Aerobes** require atmospheric O_2 (20%), and use O_2 as the final electron acceptor in the electron transport system. **Microaerophiles** require O_2 at below atmospheric concentrations, typically 2 10%. Microaerophiles have a limited ability to neutralize toxic oxygen, so excess O_2 will kill the bacteria. However, microaerophiles do use O_2 as final electron acceptor in the electron transport system. Aerobes, facultative anaerobes, and aerotolerant anaerobes must have the enzymes superoxide dismutase ($2\ O_2^{-}. + 2\ H^{+} \rightarrow O_2 + H_2O_2$) and either catalase ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$) or peroxidase ($H_2O_2 + 2\ H^{+} \rightarrow 2\ H_2O$). **Obligate Anaerobes** cannot survive in the presence of any oxygen. Obligate anaerobes lack the enzymes necessary to break down the toxic by-products of oxygen. In these bacteria, the final electron acceptor in the electron transport system is a molecule other than O_2. **Aerotolerant Anaerobes** grow equally well in the presence or absence of oxygen. They do possess enzymes necessary to neutralize toxic oxygen by-products, but they never use O_2 as a final electron acceptor. **Facultative Anaerobes** are also able to live either in the presence or absence of oxygen, but they prefer oxygen so they can carry out aerobic respiration with O_2 as final electron acceptor to maximize ATP yields like *E. coli* and some yeasts. These organisms can use other electron acceptors if O_2 is not available, such as fumarate and nitrate. They can also utilize fermentative metabolism in the absence of oxygen. One simple way to determine the oxygen requirements of a bacterium is to inoculate the bacterial culture into a melted agar deep tube of brain heart infusion (BHI) or nutrient agar media, mixing well to evenly distribute the bacteria throughout the

liquefied agar. Once the agar solidifies, an oxygen gradient is created, with atmospheric oxygen concentrations at the top of the tube and little to no oxygen present at the bottom of the tube. The bacteria present in the agar deep tube will only be able to grow where their oxygen requirements are met, and will localize to the area(s) of their oxygen requirements in the tube. Based on your knowledge of oxygen requirements, sketch where you think growth would occur in the BHI tube for each oxygen class.



REQUIREMENTS

MEDIA NEEDED:

4 Nutrient Agar or Brain Heart Infusion (BHI) deeps

Bacterial CULTURES NEEDED (depends on availability)

PROCEDURE:

1. Obtain 4 melted BHI or NA tubes from the water bath and label with 4 of the above bacteria.

2. While the BHI tube is still liquefied, add 2 drops of the appropriate inoculum into the BHI tube using a sterile Pasteur pipette. Alternatively, you may gently inoculate the

liquefied BHI tube using a sterile loop or needle. Place cap loosely (but securely) on the tube. You need to work quickly so the agar doesn't solidify!

3. Roll the BHI tube between the palms of your hands to disperse the bacteria throughout the tube, taking care not to create bubbles or aerate the agar.

4. Place the BHI tube at room temperature until solidified.

5. Once solidified, place in incubator. Incubate at 37 °C for 48 hours.

RESULTS:

Examine the BHI deep tubes for presence/absence of microbial growth and location of growth and record results in the table.

	a. Obligate Aerobes	b. Facultative Anaerobes	c. Obligate Anaerobes	d. Aerotolerant Anaerobes	e. Micro-aerophiles
Effect of Oxygen on Growth	Only aerobic growth; oxygen required.	Both aerobic and anaerobic growth; greater growth in presence of oxygen.	Only anaerobic growth; ceases in presence of oxygen.	Only anaerobic growth; but continues in presence of oxygen.	Only aerobic growth; oxygen required in low concentration.
Bacterial Growth in Tube of Solid Growth Medium					
Explanation of Growth Patterns	Growth occurs only where high concentrations of oxygen have diffused into the medium.	Growth is best where most oxygen is present, but occurs throughout tube.	Growth occurs only where there is no oxygen.	Growth occurs evenly; oxygen has no effect.	Growth occurs only where a low concentration of oxygen has diffused into medium.
Explanation of Oxygen's Effects	Presence of enzymes catalase and superoxide dismutase (SOD) allows toxic forms of oxygen to be neutralized; can use oxygen.	Presence of enzymes catalase and SOD allows toxic forms of oxygen to be neutralized; can use oxygen.	Lacks enzymes to neutralize harmful forms of oxygen; cannot tolerate oxygen.	Presence of one enzyme, SOD, allows harmful forms of oxygen to be partially neutralized; tolerates oxygen.	Produce lethal amounts of toxic forms of oxygen if exposed to normal atmospheric oxygen.

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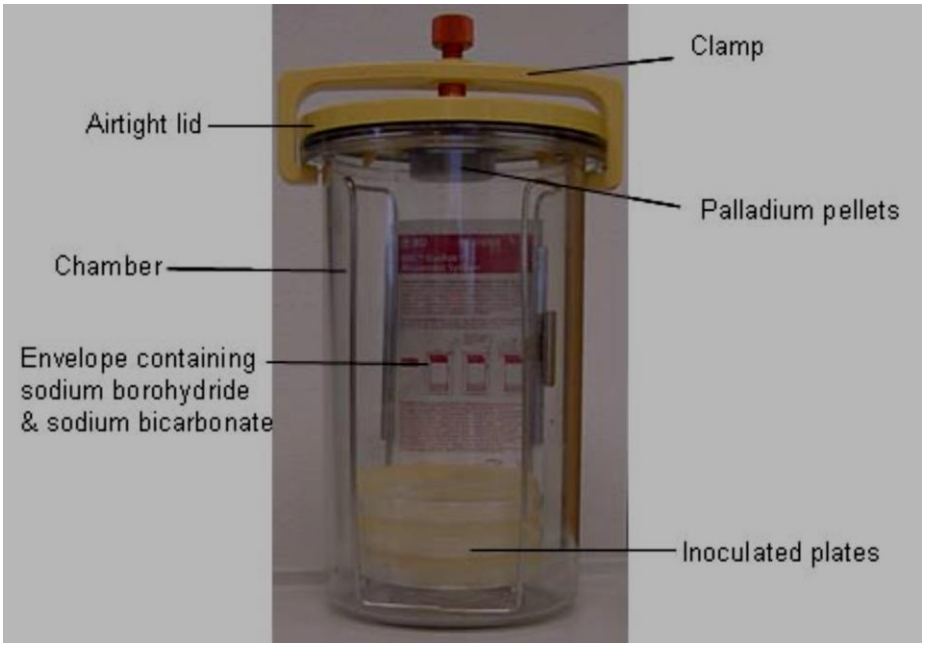
Figure 7- The effect of oxygen on various groups of bacteria.

EXPERIMENT 8 – CULTIVATION OF ANAEROBIC ORGANISMS

Specialized methods are necessary to culture organisms **anaerobically**. One such method is the use of **fluid thioglycollate broth**, which is a reducing medium. It contains **sodium thioglycollate**, which reacts with molecular oxygen keeping free oxygen levels low. The sodium thioglycollate in the broth creates a **redox potential** in the tube, with higher levels of oxygen at the top of the tube, and a complete absence of oxygen at the bottom of the tube. Fluid thioglycollate broth also typically contains a redox potential indicator such as resazurin, which produces a pink coloration in an oxidized environment. As with the BHI media described in Experiment 7, organisms will only be able to grow where their oxygen requirements are met, and will localize to the area(s) of their oxygen requirements in the fluid thioglycollate broth

A second method used to culture organisms anaerobically is the use of a **GasPak Jar**.



This is a specialized culture vessel in which an anaerobic environment is generated after inoculated media are sealed into the chamber. Anaerobic conditions are created by adding water to a gas generator envelope that is placed in the jar just before sealing. There are two chemical tablets in the envelope, **sodium borohydride** and **sodium**

bicarbonate. Water reacts with these chemicals, producing **hydrogen** gas from the sodium borohydride and **carbon dioxide** from the sodium bicarbonate. The hydrogen gas combines with free oxygen in the chamber to produce water, thus removing all free oxygen from the chamber. This reaction is catalyzed by the element **palladium** (#46 on the periodic table), which is attached to the underside of the lid of the jar. The carbon dioxide replaces the removed oxygen, creating a completely anaerobic environment.

For this lab exercise, each group will design and conduct an experiment to determine the oxygen classification of four different species of bacteria, using the provided media and equipment.

MEDIA AVAILABLE: (*per group of four)*

4 thioglycollate broth tubes

2 nutrient agar plates

EQUIPMENT AVAILABLE:

Gas Pak Jar

Incubators set at 37 °C

PROCEDURE: Design the experiment by yourself.

STUDY QUESTIONS:

1. At the molecular level, why are strict aerobes **unable** to grow in the **absence of oxygen**?
2. Since humans require oxygen in cellular respiration, we are classified as aerobic organisms. Therefore, how are obligate anaerobes such as *Clostridium tetani* (causative agent of tetanus) and *Clostridium difficile* (causes diarrhea) able to infect the human body?

EXPERIMENT 9- USE OF SELECTIVE, DIFFERENTIAL, AND ENRICHED MEDIA

Microbiologists often used multiple types of media to cultivate microorganisms in order to either specifically grow a particular organism, or to obtain information about the biochemical properties of the organisms that grow. Three commonly used types of media are selective media, differential media, and enriched media.

Selective media is designed to suppress the growth of some microorganisms while allowing the growth of others (i.e., it *selects* for certain microbes). Solid media is usually employed with selective media so that individual colonies may be isolated. Examples of selective media include mannitol salt agar, MacConkey agar, Eosin-Methylene Blue agar, and Columbia C-CNA agar.

Differential media allow the growth of more than one microorganism of interest but with morphologically distinguishable colonies based on specific biochemical properties of the organisms. Most differential media contain a substrate and a chemical indicator, often a pH indicator. As with selective media, solid media is usually employed so individual colonies may be observed. Examples of differential media include mannitol salt agar, DNAse agar, blood agar, MacConkey agar and eosin-methylene blue agar.

Enriched media contains specific growth factors needed by fastidious bacteria to support their growth. Examples of enriched media include blood agar and chocolate agar. Described below are the specific properties of some of the more commonly used selective, differential, and enriched media. Note that a particular type of media can have multiple properties – e.g. it can be both selective and differential!

Mannitol Salt Agar: Mannitol salt agar (MSA) is both selective and differential. MSA contains 7.5% NaCl, which selects for halotolerant organisms such as members of the *Staphylococcus* genus. The media also contains the carbohydrate substrate mannitol,

and a pH indicator, phenol red. Organisms which are able to ferment the mannitol will produce acid fermentation products which lower the pH, causing the phenol red indicator to turn yellow.

Blood Agar: Blood agar is a differential medium. It is also commonly used as an enriched medium for growing fastidious bacteria. Some bacteria produce exotoxins called hemolysins that cause lysis of red blood cells. The degree of the hemolysis is an especially useful tool for differentiation among Gram-positive cocci. The three types of hemolysis are:

1. **Beta hemolysis**, which is the **complete lysis** of red blood cells and hemoglobin. This results in complete clearing of the blood around colonies.

2. **Alpha hemolysis** refers to the **partial lysis** of red blood cells and hemoglobin. This results in a greenish-grey or greenish-yellow discoloration of the blood around the colonies.

3. No hemolysis, sometimes called **gamma hemolysis** results in no change in the medium.

MacConkey Agar: MacConkey Agar is both selective and differential. This media contains crystal violet and bile salts, which inhibit most gram-positive organisms and select for gram-negative organisms. It also contains the substrate lactose and the pH indicator neutral red, which allow differentiation among gram-negative bacteria based on their ability to ferment lactose. When lactose is fermented by coliforms such as *Escherichia coli*, acid end-products lower the pH of the media below 6.8, with the resulting colonial growth turning pinkish-red. If an organism is unable to ferment lactose, the colonies will be colorless, taking on the color of the medium.

Eosin Methylene Blue Agar: Eosin Methylene Blue (EMB) agar is both selective and differential. This media contains the dyes eosin and methylene blue, which inhibit gram-

positive growth and select for gram-negative organisms. It also contains lactose, allowing differentiation between organisms which ferment lactose and produce acid end-products, and organisms that do not ferment lactose. Small amounts of acid production result in a pink colored growth (e.g. *Enterobacter aerogenes*) while large amounts of acid cause the acid to precipitate on the colony, resulting in a characteristic greenish, metallic sheen (e.g. *E. coli*). Organisms which do not ferment lactose will be colorless, taking on the color of the medium.

Columbia C-NA Agar: Columbia C-NA (CCNA) agar is both selective and differential. This media contains two antibiotics, colistin and nalidixic acid, which prohibit the growth of most gram-negative organisms. Gram-positive organisms such as members of the *Staphylococcus* and *Streptococcus* genera are allowed to grow. Addition of blood to the agar allows differentiation by hemolytic patterns.

MEDIA NEEDED:

1 Mannitol Salt agar plate

1 Blood agar plate

1 MacConkey agar plate

1 Eosin-Methylene Blue (EMB) agar plate

1 Columbia C-NA (CCNA) agar plate

CULTURES NEEDED:

Enterobacter aerogenes, Escherichia coli, Pseudomonas aeruginosa, Streptococcus zooepidemicus, Streptococcus mutans, Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Salmonella typhimurium

PROCEDURE:

1. Label each plate according to the diagram on the following page. Don’t forget to also put your name/initials and the date.

2. Using a sterile loop, inoculate each one of the plates using a straight line streak .

3. Tape all plates on both sides.

4. Place the blood agar plates and CCNA plates into a GasPak jar or Anaeropak box so they can be incubated in an environment enriched with carbon dioxide.

5. Incubate all plates at 37 °C for 48 hours.

RESULTS:

Examine plates for the following:

1. Mannitol Salt Agar Plates: Growth on mannitol salt agar indicates the organism is halotolerant. A change in the color of the agar from pink to yellow indicates the organism has the ability to ferment mannitol. Note: this yellow coloration can usually be seen around the sides of the bacterial growth.

2. Blood Plates: All organisms should grow on the blood plates – they are NOT selective. Growth surrounded by a complete clearing of the blood indicates beta hemolysis. Growth surrounded by a partial clearing of the blood (often a greenish-grey color) indicates alpha hemolysis. Growth which produces no changes in the blood indicates the bacteria are non-hemolytic. (This lack of hemolysis is sometimes called gamma hemolysis.)

3. MacConkey Agar Plates: Growth on MacConkey agar indicates the organism is resistant to crystal violet and bile salts, and is likely to be gram-negative. Growth which is a pinkish-red color indicates the organism has the ability to ferment lactose, and is probably a coliform.

4. Eosin Methylene Blue Agar: Growth on Eosin Methylene Blue agar indicates the organism can grow in the presence of the dyes eosin and methylene blue and is likely a gram-negative. Growth which is a pink color indicates the organism can ferment lactose to form weak acid end-products, and growth which exhibits a green metallic sheen indicates the organism can ferment lactose to form strong acid end-products.

5. C-CNA Agar: Growth on Columbia C-NA agar indicates the organism is resistant to the antibiotics colistin and naladixic acid, and is likely a gram-positive. Hemolysis patterns can allow differentiation.

Record your results on the data charts below, one is given as an example. On blood agar and C-CNA agar, pattern of hemolysis is recorded instead of color.

Media: Mannitol Salt Agar

Organism	Growth	Color	Interpretation of Results
Streptococcus mutans	+/-ve	Pink/Yellow	name of expected bacteria
Staphylococcus epidermidis			
Staphylococcus aureus			
Enterococcus faecalis			

EXPERIMENT 10- CATALASE TEST

Staphylococcus species produce an enzyme called *catalase*, which reacts with the toxic oxygen by-product hydrogen peroxide (H_2O_2), breaking it down into water (H_2O) and molecular oxgyen (O_2).

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2$$

Streptococcus and *Enterococcus* species do not produce *catalase* (although they produce *superoxide dismutase* and *peroxidase*). Therefore, the *catalase* test is widely used to distinguish *Staphylococcus* from *Streptococcus* and *Enterococcus.*

CULTURES NEEDED: *Staphylococcus* and *Streptococcus/Enterococcus.*

PROCEDURE:

1. Drop 2 small drops of 3% hydrogen peroxide onto a clean glass slide. Using a sterile loop, put a loop full of the *Staphylococcus* organism into one of the drops. Observe for a bubbling reaction. (The bubbles are the molecular oxygen that is produced as the *catalase* breaks down the hydrogen peroxide.)

Catalase-positive: bubbles observed **Catalase-negative:** no bubbles

2. Repeat with *Streptococcus* organism using a sterile loop to put a loop full of the bacteria into the other drop of hydrogen peroxide.

RESULTS:

Organism: _____ Catalase Reaction: **+** or **–** *(circle one)*

STUDY QUESTIONS:

1. In attempting to distinguish between *Staphylococcus* and *Streptococcus* species, what is the ***first*** lab test that should be performed? Why?

2. Streptococcus and *Enterococcus* species do not produce the enzyme *catalase*. How are these aerotolerant bacteria able to survive in the presence of oxygen when they lack this important enzyme?

Experiment 11- Hanging-Drop and Wet-Mount Preparations

PURPOSE: To observe bacteria in a hanging drop, study their morphology, and determine their motility

MATERIALS: 24-hour broth culture of *Proteus vulgaris* mixed with a light suspension of yeast cells, 24-hour broth culture of *Staphylococcus epidermidis* mixed with a light suspension of yeast cells, 2 hollow-ground slides, several cover glasses, wire loop, Bunsen burner or bacterial incinerator, Permanent marking pen, Petroleum jelly.

PRINCIPLE: The simplest method for examining living microorganisms is to suspend them in a fluid (water, saline, or broth) and prepare either a “hanging drop” or a simple “wet mount.” The slide for a hanging drop is ground with a concave well in the center; the cover glass holds a drop of the suspension.

When the cover glass is inverted over the well of the slide, the drop hangs from the glass in the hollow concavity of the slide. Microscopic study of such a wet preparation can provide useful information. Primarily, the method is used to determine whether or not an organism is motile, but it also permits an undistorted view of natural patterns of cell groupings and of individual cell shape. Hanging-drop preparations can be observed for a fairly long time, because the drop does not dry up quickly. Wet-mounted preparations are used primarily to detect microbial motility rapidly. The fluid film is thinner than that of hanging-drop preparations and therefore the preparation tends to dry up more quickly, even when sealed. Although the hanging drop is the classical method for viewing unstained microorganisms, the wet mount is easier to perform and usually provides sufficient information.

PROCEDURE 1. A thin film of petroleum jelly is placed around the concave well on the hollow-ground slide.

2. Take a cover glass and clean it thoroughly, making certain it is free of grease (the drop to be placed on it will not hang from a greasy surface). It may be dipped in alcohol and polished dry with tissue, or washed in soap and water, rinsed completely, and wiped dry.

3. Using aseptic technique, sterilize the wire loop, remove the cap of the tube, and take up a loop full of culture. Be certain the loop has cooled to room temperature before inserting it into the broth or it may cause the broth to “sputter” and create a dangerous aerosol. Close and return the tube to the rack.

4. Place the loop full of culture in the center of the cover glass (do not spread it around). Sterilize the loop and put it down.

5. The hollow-ground slide is inverted over the drop on the cover glass. After correct positioning, the slide is pressed gently against the cover glass to seal them together with the petroleum jelly.

6. The hollow-ground slide is re-inverted so that the drop of suspension now hangs from the cover glass in the concave well of the slide. You should have a sealed wet mount, with the drop of culture hanging in the well.

7. Place the slide on the microscope stage, cover glass up. Start your examination with the low-power objective to find the focus. It is helpful to focus first on one edge of the drop, which will appear as a dark line. The light should be reduced with the iris diaphragm and, if necessary, by lowering the condenser. You should be able to focus easily on the yeast cells in the suspension.

7. Continue your examination with the high-dry and oil-immersion objectives (be very careful not to break the cover glass with the latter). Although the yeast cells will be obvious because of their larger size, look around them to observe the bacterial cells.

9. Record your observations of the size, shape, cell groupings, and motility of the two bacterial organisms in comparison to the yeast cells.

Note: True, independent motility of bacteria depends on their possession of flagella. If so equipped, they can propel themselves with progressive, directional locomotion (often quite rapidly). This kind of active motion must be distinguished from the vibratory movement of organisms or other particles suspended in a fluid. The latter type of motion is called *Brownian movement* and is caused by the continuous, rapid oscillation of molecules in the fluid. Small particles of any kind, including bacteria (whether motile or not), are constantly bombarded by the vibration of the fluid molecules, and so are bobbled up and down, back and forth. Such movement is irregular and non-directional and does not cause non motile organisms to change position with respect to other objects around them. You must be careful not to mistake movement caused by currents in a liquid for true motility. If a wet mount is not well sealed or contains bubbles, air currents set up reacting fluid currents, and you will see organisms streaming along on a tide.

Results

1. Make drawings in the following circles to show the *shape* and *grouping* of each organism. Indicate below the circle whether it is *motile* or *nonmotile.* How does their size compare with that of the yeast cells in the preparation?

PROCEDURE Preparing a Wet Mount

Purpose To observe bacteria in a simple wet mount and determine their motility

Materials 24-hour broth culture of *Proteus vulgaris* mixed with a light suspension of yeast cells, 24-hour broth culture of *Staphylococcus epidermidis* mixed with a light suspension of yeast cells, 2 microscope slides, Several cover glasses, Capillary pipettes and pipette bulbs, permanent marking pen, Clear nail polish (optional).

Procedure: 1. Using a pipette bulb, aspirate a small amount of the *Proteus* culture with a capillary pipette and place a *small* drop on a clean microscope slide (Figure 5, step 1).

2. Carefully place a clean cover glass over the drop, trying to avoid bubble formation (fig 5, step 2). The fluid should not leak out from under the edges of the cover glass. If it does, wait until it dries before sealing.

3. If you examine the slide immediately, you need not seal the coverslip. Otherwise, seal around the edges of the coverslip with a thin film of clear nail polish (fig.5, step 3). Be certain the nail polish is completely dry before examining the slide under the microscope.

4. Examine the preparation in the same manner as above. Instead of focusing on the edge of the drop, however, you may find it helpful to focus first on the left-hand edge of the coverslip.

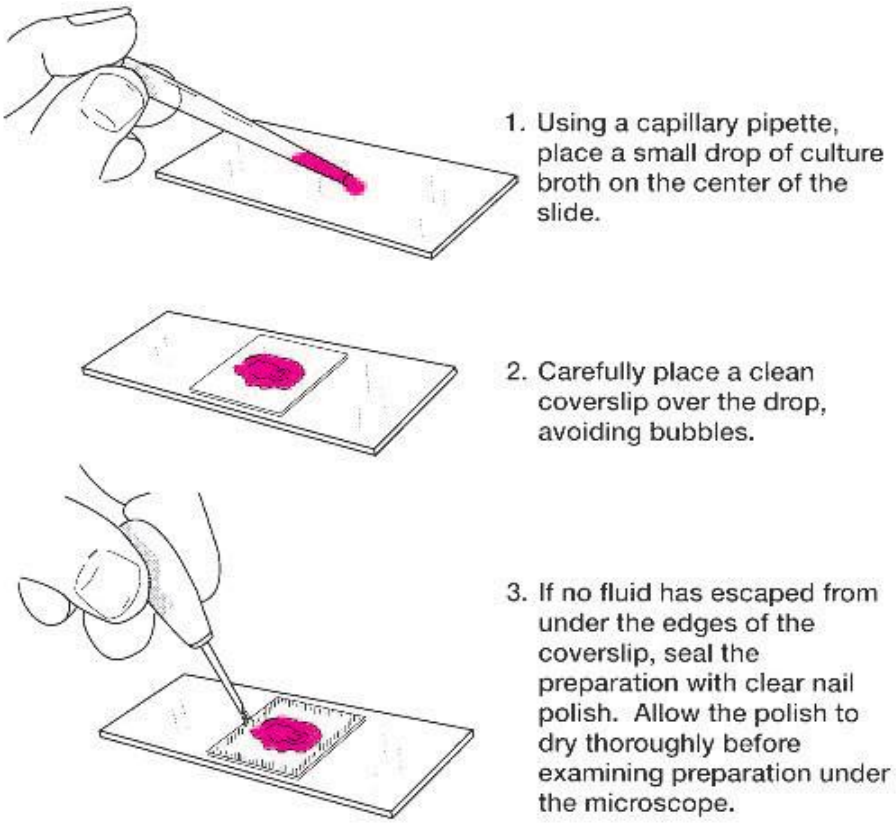

1. Using a capillary pipette, place a small drop of culture broth on the center of the slide.
2. Carefully place a clean coverslip over the drop, avoiding bubbles.
3. If no fluid has escaped from under the edges of the coverslip, seal the preparation with clear nail polish. Allow the polish to dry thoroughly before examining preparation under the microscope.

Figure 8. Wet-mount preparation.

Study Questions

1. How does true motility differ from Brownian movement?
2. What morphological structure is responsible for bacterial motility?
3. What is the value of a hanging-drop preparation?
4. What is the value of a wet-mount preparation?

Experiment 12- STAINING OF BACTERIAL SPECIMENS

An important part of the problem in the study of bacteria is their minute size. Because they are so small and have so little substance, they tend to be transparent, even when magnified in subdued light. The trick, then, is to find ways to stop their motion and tag their structures with something that will make them more visible to the human eye.

Bacterial specimens are often stained prior to microscopy studies, which allow better visualization of specimens using microscopy. Simply speaking, a stain is a substance that adheres to a cell, giving the cell color. Different stains have different affinities for different organisms, or different parts of organisms. They may be used to differentiate different types of organisms or to view specific parts of organisms.

There are several types of stains which are commonly used in microbiology. The first is a **simple stain**, which uses only one reagent which provides contrast between the background and the heat-fixed bacterium itself. The bacterium takes up stain and becomes colored, while the background remains unstained. Simple stains are typically used on bacterial smears which have been heat-fixed and thus contain non-living microbes. A second type of stain is a **negative stain**, which uses a single reagent to provide contrast between the background and the living bacterium. Thus, the background is “stained”, while bacterium does not take up any stain. Negative stains are typically used when observing live bacteria is desired.

A **differential stain** is a type of staining that allows you to distinguish between types of bacteria or between specific structures in a bacterium. A differential stain typically uses two or more reagents – a primary stain and a counter stain.

Chemically, there are two main types of stains: **basic stains**, which have a positive charge (cationic) and **acidic stains**, which have a negative charge (anionic). Basic stains have an affinity for negative components of cells, and include dyes such as methylene

blue, crystal violet, and carbol fuchsin. Acidic stains have an affinity for positive components of cells, and include dyes such as nigrosin, India ink, and picric acid. The free ions of *acidic* dyes are anions (negatively charged) that combine with cations of a base in the stained cell to form a salt. Basic dyes possess cations (positively charged) that combine with an acid in the stained material to form a salt. Bacterial cells are rich in ribonucleic acid (contained in their abundant ribosomes) and therefore stain very well with basic dyes. Since cell walls are negatively charged, a positive dye will be attracted to and stain the cell wall, whereas a negative dye will be repulsed by the cell wall and not directly stain the cell.

Stained bacteria can be measured for size and are classified by their shapes and groupings. Bacteria are so small that their size is most conveniently expressed in *micrometers* (symbol μm). A micrometer is a thousandth part of a millimeter, and 1/10,000 of a centimeter, or 1/25,400 of an inch. Bacteria vary in length and diameter, the smallest being about 0.5 to 1 μm long and approximately 0.5 μm in diameter, whereas the largest filamentous forms may be as long as 100 μm.

.Bacteria have rigid cell walls and maintain a constant shape. Therefore, they can be classified on the basis of their form. Bacteria have three basic shapes: *spherical* (round), *rod shaped,* or *spiraled.* A round bacterium is called a *coccus* (plural, *cocci*). A rod-shaped organism is called a *bacillus* (plural, *bacilli*) or simply a *rod.* A spiraled bacterium with at least two or three curves in its body is called a *spirillum* (plural, *spirilla*). Long sinuous organisms with many loose or tight coils are called *spirochetes.* The patterns formed by bacterial cells grouping together as they multiply are often characteristic for individual bacterial genera or species. Cocci may occur in pairs (*diplococci*), chains (*streptococci*), clusters (*staphylococci*), or packets of four (*tetrads*), and are seldom found singly. Rod-shaped bacteria (bacilli) generally occur as individual cells, but they may appear as end-

to-end pairs (*diplobacilli*) or line up in chains (*streptobacilli*). Some species tend to *palisade,* that is, line up in bundles of parallel bacilli, others may form V, X, or Y figures as they divide and split. Some may show great variation in their size and length (pleomorphism). Spiraled bacteria occur singly and usually do not form group patterns. Examine following figure to see representative examples of bacterial morphology.

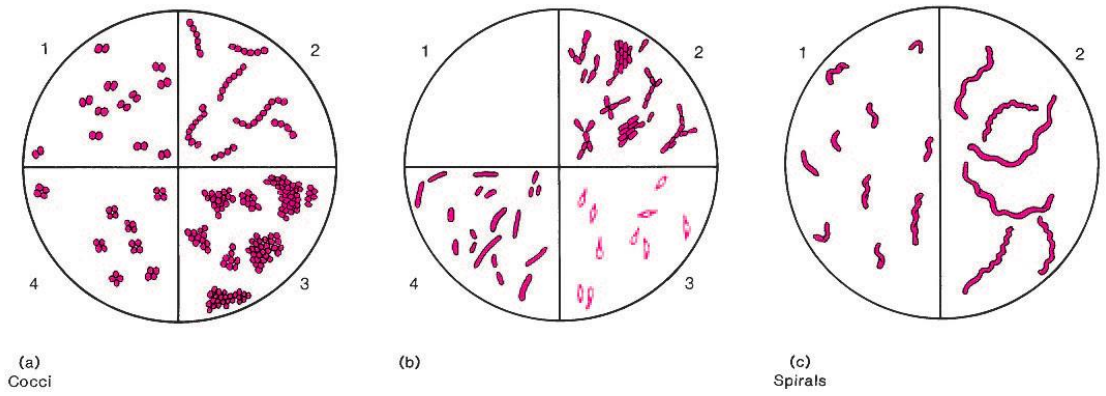


Figure 9. Basic shapes and arrangements of bacteria.

Cocci. 1. Diplococci (pairs); 2. Streptococci (chains); 3. Staphylococci (grapelike clusters); 4. Tetrads (packets of four). (b) Bacilli (rods). 1. Streptobacilli (chains); 2. Palisades; V, X, and Y figures, clubbing; 3. Endospore-forming bacilli (note endospores as small, round, hollow, unstained areas, within or at one end of bacillary bodies); 4. A bacillus showing pleomorphism (note varying widths and lengths). (c) Spirals. 1. Spirilla (short curved or spiraled forms with rigid bodies); 2. Spirochetes (long tightly or loosely coiled forms with sinuous flexible bodies).

PREPARATION OF BACTERIAL SPECIMENS

In order to stain bacterial specimens, the bacteria must be placed on a glass slide and prepared for the specific staining process. This is typically done by preparing a **bacterial smear**. A smear is a small volume (a loopful) of specimen-containing medium that is spread (smeared) onto a microscope slide. In preparing smears, everything has to be done just right! If smears are too thick, you will have trouble seeing individual cells. If smears are too thin, you may not find the organism. If you stir the drop of medium too much as you spread it on the slide, you will disrupt cell arrangements such as chains or clusters. Following preparation of a bacterial smear, most smears need to be heat fixed before they are stained. Heat fixation accomplishes three things: (1) it kills the organisms; (2) it causes the organisms to adhere to the slide; and (3) it alters the organisms so that they more readily accept stains (dyes). Again, the Goldilocks principle applies. Slides must be completely dry before heat-fixing, or the organism will be boiled and destroyed. If you heat-fix too little, the organism will wash off the slide. If you heat-fix too much, the organisms may be incinerated.

PROCEDURE FOR PREPARING A BACTERIAL SMEAR

1. Obtain a glass slide and clean if necessary.

2. Using a **broth culture**:

a. Gently agitate your culture broth tube to disperse the bacteria.

b. Sterilize your inoculating loop using an incinerator or a Bunsen burner, and let cool for 20-30 seconds.

c. Place loop in the bacterial broth and put the loopful of the broth onto the glass slide. Rub the drop into a nickel-sized smear. Sterilize the loop again to kill any remaining bacteria. Let the smear **air** dry completely. Do **not** use heat to dry your smear!

3. Using an **agar plate**:

a. Place a **small** drop of water in the center of the slide.

b. Sterilize your inoculating loop using an incinerator or a Bunsen burner, and let cool for 20-30 seconds.

c. Use the sterile loop to pick up a small amount of bacterial growth from the surface of the plate. Do not dig into the agar. Put the loopful of bacteria into the drop of water on the glass slide, and rub the drop into a nickel-sized smear.

Sterilize the loop again to kill any remaining bacteria Let the smear **air** dry completely. Do **not** use heat to dry your smear!

4. **Heat fix** the slide.

a. **Incinerator method:** Hold the slide with a wooden clothes pin approximately 1 cm over the barrel of a hot incinerator for 20–30 seconds. Let the slide cool.

b. **Bunsen burner method:** Hold the slide with a wooden clothes pin, and pass 10- 12 times through the flame. Let the slide cool.

5. **Optional:** After the slide has cooled us a marker or wax pencil to outline the area of the smear on the underside of the slide. This will help you locate your sample later.

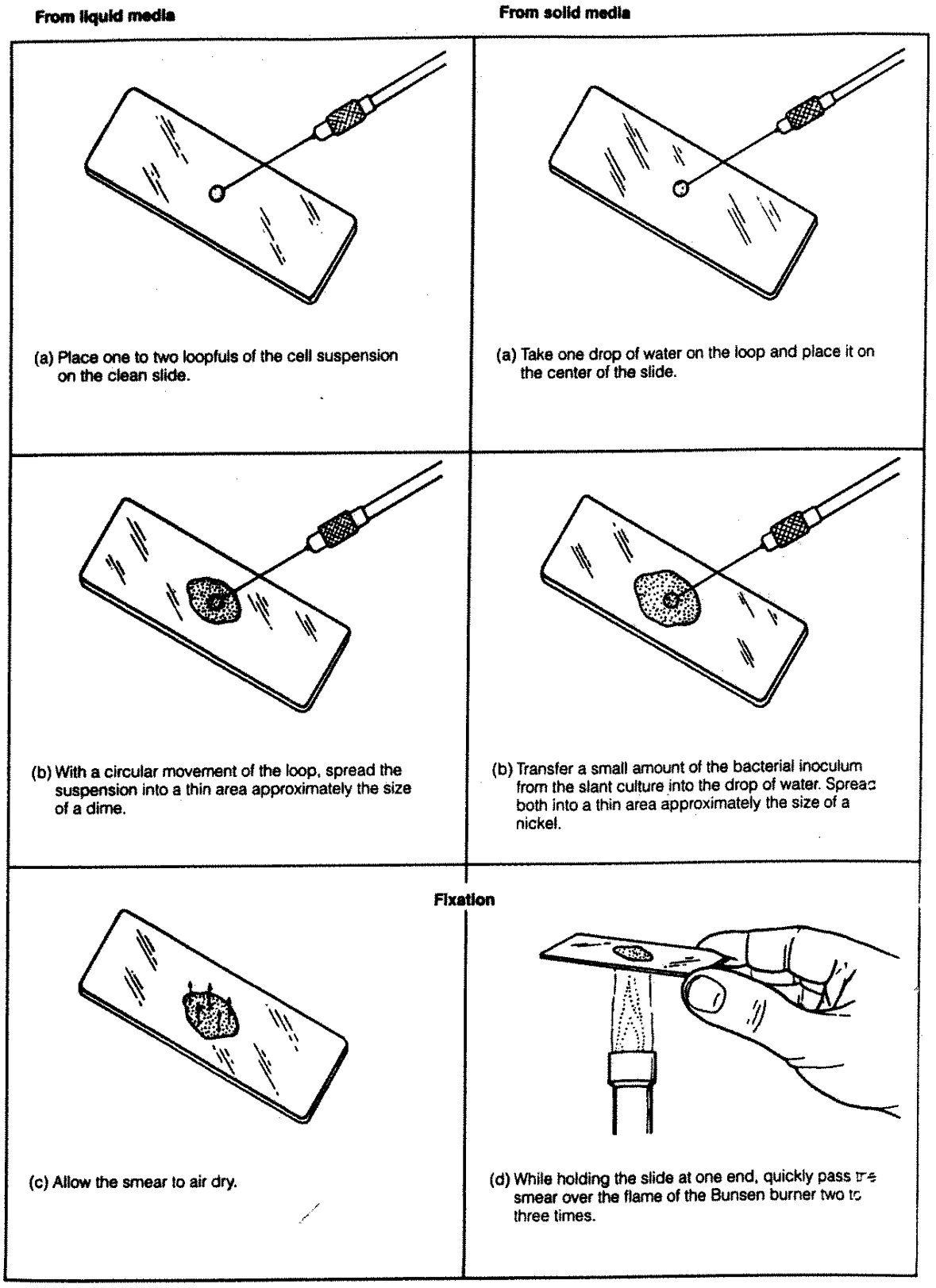

From liquid media
From solid media
(a) Place one to two loopfuls of the cell suspension on the clean slide.
(a) Take one drop of water on the loop and place it on the center of the slide.
(b) With a circular movement of the loop, spread the suspension into a thin area approximately the size of a dime.
(b) Transfer a small amount of the bacterial inoculum from the slant culture into the drop of water. Spread both into a thin area approximately the size of a nickel.
Fixation
(c) Allow the smear to air dry.
(d) While holding the slide at one end, quickly pass the smear over the flame of the Bunsen burner two to three times.

Figure 10. Smear formation from liquid and solid culture medium.

EXPERIMENT -13 GRAM STAINING

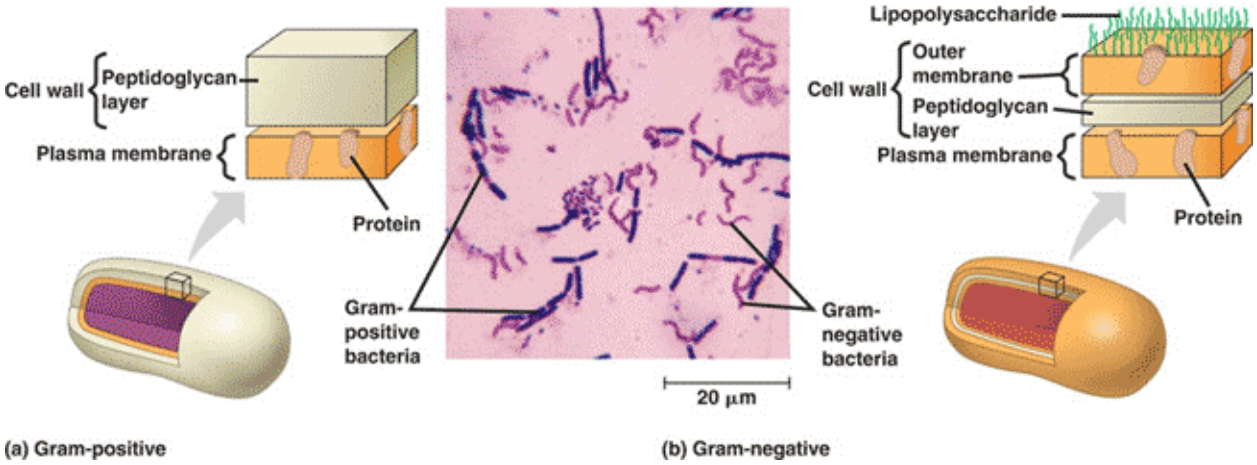
The **Gram stain** is a differential stain which distinguishes bacteria based on cell wall properties. Bacterial cell walls are composed primarily of **peptidoglycan** and bacteria can be classified into two main groups dependent on the amount of peptidoglycan present in their cell wall. **Gram-positive** organisms have a thick layer of peptidoglycan, whereas **Gram-negative** organisms have a thin layer of peptidoglycan, plus an additional **outer membrane** that is absent in Gram-positive organisms.

In the gram staining procedure, the **primary stain** is crystal violet, and all cells take up the purple crystal violet stain. Following the primary stain, Gram's Iodine is applied to the bacterial smears. The iodine acts as a **mordant,** enhancing the ability of the stain to enter and bind to the bacteria. Specifically, the iodine binds with crystal violet and locks it into peptidoglycan of bacteria. It also intensifies the purple color. The **decolorizing agent** used in the gram staining procedure is 95% ethanol, which is a lipid solvent that melts the Gram negative outer membrane and leads to decolorization of Gram-negative cells. It also dehydrates proteins, helping the primary stain to remain in Gram-positive cell walls. The **counter stain** then used is Safranin, which stains the decolorized Gram-negative cells pink. Thus, at the end of the staining procedure, Gram-positive cells are purple and Gram-negative cells are pink.

Note: It is preferable to use fresh cultures for the Gram stain. Old cultures may stain "Gram-variable (a mix of purple and pink) because they decolorize easily.

Overview of Gram Stain:

Primary Stain	Crystal Violet
Mordant	Gram's Iodine
Decolorizer	95% Ethanol
Counter Stain	Safranin



CULTURES NEEDED:

Nutrient broth tubes or plates of the following cultures or any other available in lab:

Escherichia coli

Staphylococcus aureus

PROCEDURE:

Note: This is an individual lab exercise – each student will perform their own Gram stain.

1. Prepare a bacterial smear with a mixture of all 3 organisms (i.e. 1-2 loopful of each) listed above and **heat fix** as described on page 13.

2. Place the slide on a staining tray, and cover the smear with crystal violet. Allow to stain for 60 seconds.

3. Tilt the slide and gently rinse with distilled water until the stain is removed.

4. Cover the smear with Gram's Iodine, and allow to sit for 60 seconds.

5. Tilt the slide and gently rinse with distilled water.

6. **IMPORTANT STEP**: Tilt the slide and let 2-3 drops of Decolorizer run over the slide. If the last drop is still purple, continue decolorizing, 2-3 drops at a time, until the decolorizer runs clear. Rinse with distilled water.

7. Cover the smear with Safranin, and stain for 45 seconds.

8. Tilt the slide and rinse with distilled water.

9. Place the slide in a book of bibulous paper and blot to dry. You do not need a cover slip!

Observe the slide under oil immersion, and draw what you see in the results section below. You should see: Small purple cocci (spheres) which are the gram-positive and small pink rods, which are the gram-negative *E. coli*. **Label** these in your drawing.

10. Clean your microscope with lens cleaner, paying extra attention to the 40X and 100X objectives.

11. Dispose of staining waste in designated waste receptacle. Dispose of slides in SHARPS container.

RESULTS:

Using colored pencils, draw and label *S. aureus* and *E. coli.*

STUDY QUESTIONS:

1. Describe several advantages of differential staining procedures compared with simple staining techniques.
2. Give the purpose of each of the following reagents in a differential staining procedure:

a. Primary stain

b. Counter stain

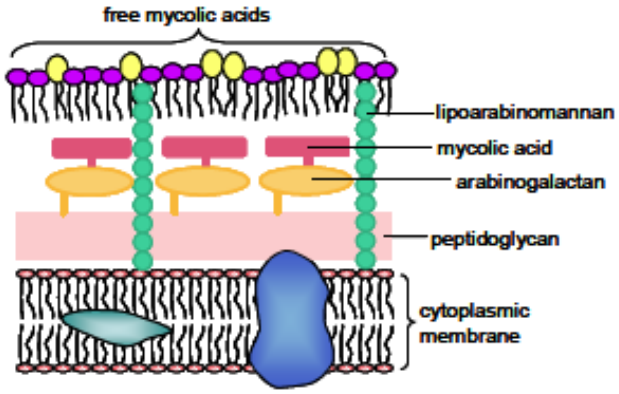
c. Decolorizing agent

d. Mordant

3. Why is it important for the counter stain to be a lighter color than the primary stain?

EXPERIMENT -14 ACID FAST STAINING

The **acid-fast stain** is a differential stain which distinguishesbacteria based on cell wall properties. Bacteria which are acid-fast are Gram-positive microorganisms that are resistant to the Gram stain, so implementing the acid-fast stain allows us to visualize these bacteria. Acid-fast bacteria have a waxy substance called **mycolic acid** in their cell walls, which comprises up to 60% of the cell wall components. They also contain polymers called arabinogalactan and lipoarabinomannan. Genera which are acid-fast include *Mycobacterium* and *Nocardia*, which include pathogenic organisms such as *M. leprae* and *M. tuberculosis*.



Acid-Fast Cell Wall

The **primary stain** in the acid-fast staining procedure is **carbol fuchsin**, which contains **phenol** to solubilize the cell wall and allow the stain to enter the cell. The procedure requires the application of **heat** in order for the stain to penetrate the waxy cell wall. All cells will take up the reddish-pink primary stain. Once the cytoplasm is stained, it resists decolorization, even with harsh agents such as acid-alcohol, which cannot dissolve and penetrate beneath the mycobacterial lipid wall. Under these conditions of staining, the mycobacteria are said to be *acid fast* Once the cells have been allowed to cool after the primary stain, the cells are decolorized. The **decolorizer** used in the acid-fast staining procedure is **acid alcohol**, which decolorizes all cells except acid-fast cells. The acid

alcohol is unable to penetrate the waxy cell wall of acid-fast microorganisms. The **counterstain** then used is **methylene blue**, which stains the decolorized non acid-fast cells blue. Thus, at the end of the staining procedure, acid-fast cells are reddish-pink and all other cells are blue and are said to be *nonacid fast*.

PROCEDURE

1. Prepare two fixed smears of each culture and two of the simulated sputum. In practice, the smears are fixed with methanol for one minute or are heat-fixed at 65 to 75°C to be certain any tuberculosis bacilli present are killed. To make smears of the agar slant culture, first place a drop of water on the slide, and then emulsify a small amount of the colonial growth in this drop.

2. Ring and code one slide of each pair with your marking pencil or pen, as usual.

3. The other slide of each pair must be ringed and coded with a diamond pencil. This device scratches the glass indelibly, so that the marks remain even during the prolonged staining process.

4. Cover smear with a 2 _ 3-cm piece of filter paper to hold the stain on the slide and to filter out any undissolved dye crystals. Flood the slide with concentrated carbolfuchsin solution and allow to stand for five minutes.

5. Use forceps to remove filter paper strips from slides and place the strips in a discard container. Rinse slides with water and drain.

6. Cover smears with acid-alcohol solution and allow them to stand for two minutes.

7. Rinse again with water and drain.

8. Flood smear with methylene blue and counterstain for one to two minutes.

9. Rinse, drain, and air dry.

10. Examine all slides under oil immersion and record observations under Results.

EXPERIMENT -15 SPECIAL STAINS

Some bacteria have characteristic surface structures (such as *capsules* or *flagella*) and internal components (e.g., *endospores*) that may have taxonomic value for their identification. When it is necessary to demonstrate whether or not a particular organism possesses a capsule, is flagellated, or forms endospores, special staining techniques must be used.

Many bacterial species possess an exterior capsule composed of carbohydrate or glycoprotein. A few pathogenic species, such as *Streptococcus pneumoniae* (the leading cause of bacterial pneumonia) and *Klebsiella pneumoniae* (a cause of pneumonia and wound and urinary tract infections) have well-developed capsules that contribute to virulence by preventing phagocytic cells from ingesting and killing the bacteria. Capsules do not retain staining agents, but can be made visible microscopically by the use of a simple, nonspecific *negative staining technique.* A small drop of India ink or nigrosin is added to a suspension of bacterial cells on a glass slide. These agents do not penetrate the cells (or stain the surrounding capsules), but serve as background stains, which outline the capsules. When the slide is dry, the preparation is stained with safranin, which penetrates and stains the cells. After this treatment, the bacteria appear pink and their capsules stand out sharply as clear, unstained zones against the dark background of India ink or nigrosin. In the animal body, carbohydrate or protein capsular components are recognized as foreign substances (referred to as antigens). In response to the presence of capsular antigens, antibodies are produced that react with (bind to) the capsule. The binding of antibodies to antigen (called an antigen-antibody reaction) greatly enhances phagocytosis. Many variations in the chemical structure of capsular antigens exist, even within a single bacterial species, and each variation stimulates the production

of antibodies specific for that capsular type. For example, more than 80 capsular types of *S. pneumoniae* have been identified. serological tests, which make use of these specific antigen-antibody reactions, are often used for rapid and accurate identification of bacteria present in clinical specimens. Encapsulated bacteria may be identified serologically by one such test, called the *quellung reaction.* In this test, unknown bacteria from the clinical specimen are placed on a slide and antiserum (serum containing known antibodies) is added. When the preparation is viewed under the microscope, if an antigen-antibody reaction has occurred between antibodies in the test serum and capsular antigens on the bacterial cells (a positive test), the capsules become much more distinct and appear to swell. Bacterial flagella are tiny hairlike organelles of locomotion. Originating in the cytoplasm beneath the cell wall, they extend beyond the cell, usually equaling or exceeding it in length. Their fine protein structure requires special staining techniques for demonstrating them with the light microscope. Since not all bacteria possess flagella, their presence, numbers, and pattern or arrangement on the cell may provide clues to identification of species. For example, *Vibrio cholera* and some species of *Pseudomonas* have a single polar flagellum at one end of the cell (they are said to be *monotrichous*), some spirillae display bipolar tufts of flagella (the arrangement is called *lophotrichous*), while many *Proteus* species have multiple flagella surrounding their cells (in a *peritrichous* pattern). Some flagellar stains employ rosaniline dyes and a mordant, applied to a bacterial suspension fixed in formalin and spread across a glass slide. The formalin links to, or “fixes,” the flagellar and other surface protein of the cells. The dye and mordant then precipitate around these “fixed” surfaces, enlarging their diameters, and making flagella visible when viewed under the microscope. In another method, a ferric-tannate mordant and a silver nitrate solution are applied to a bacterial suspension. The resulting dark precipitate that forms on the bacteria and their flagella allows them to be easily visualized

under the microscope. This silver-plating technique is also used to stain the very slender spirochetes. Among bacteria, endospore formation is most characteristic of two genera, *Bacillus* and *Clostridium.* The process of sporulation involves the condensation of vital cellular components within a thick, double-layered wall enclosing a round or ovoid inner body. The activities of the vegetative (actively growing) cell slow down, and it loses moisture as the endospore is formed. Gradually, the empty bacterial shell falls away. The remaining endospore is highly resistant to environmental influences, representing a resting, protective stage. Most disinfectants cannot permeate it, and it resists the lethal effects of drying, sunlight, ultraviolet radiation, and boiling. It can be killed when dry heat is applied at high temperatures or for long periods, by steam heat under pressure (in the autoclave), or by special sporicidal (endospore-killing) disinfectants. Because bacterial endospore walls are not readily permeated by materials in solution, the inner contents of the endospores are not easily stained by ordinary bacterial dyes. When sporulating bacteria are Gram stained, the endospores forming within the vegetative cells appear as empty holes in the bacterial bodies. Depending on their location within the cell, the endospores are referred to as terminal (at the very end of the vegetative cell), subterminal (near, but not at, the end of the cell), or central. Free endospores are invisible when stained with the Gram stain or appear as faint pink rings. To demonstrate the inner contents of bacterial endospores, you must use a special staining technique that can drive a dye through the endospore coat.

SPORE STAINING

Purpose To learn a technique for staining bacterial endospores

Materials 3- to 5-day-old agar slant culture of *Bacillus subtilis*

24-hour-old slant culture of *Staphylococcus epidermidis*

Malachite green solution

Safranin solution

Slides

Diamond glass-marking pencil

Slide rack

500-ml beaker

Tripod with asbestos mat

Forceps

Procedures

1. Place a drop of water on a slide. Emulsify a small amount of each of the slant cultures in the same drop of water.

2. Ring and code the slide with the diamond marking pencil.

3. Stain the slide by the endospore stain:

a. Place the slides on a slide rack extended across a beaker of boiling water held on a tripod (an electric burner may be

used instead of a Bunsen burner). An asbestos mat should protect the beaker from the Bunsen flame beneath (fig. 7.2).

b. Flood slide with malachite green and allow to steam gently for 5 to 10 minutes. The stain itself should not boil; if it does,

reduce the heat. If the stain appears to be evaporating and drying too rapidly, add a little more. Keep the slide flooded.

c. Allow the slide to cool slightly, then use forceps to drain the slide over a sink or staining tray and rinse with water for

about 30 seconds until no more green washes out.

d. Counterstain the preparation with safranin for 30 seconds, then rinse again with tap water. Blot or air-dry the slide.

e. Examine the smear under the oil-immersion objective and record your observations under Results.

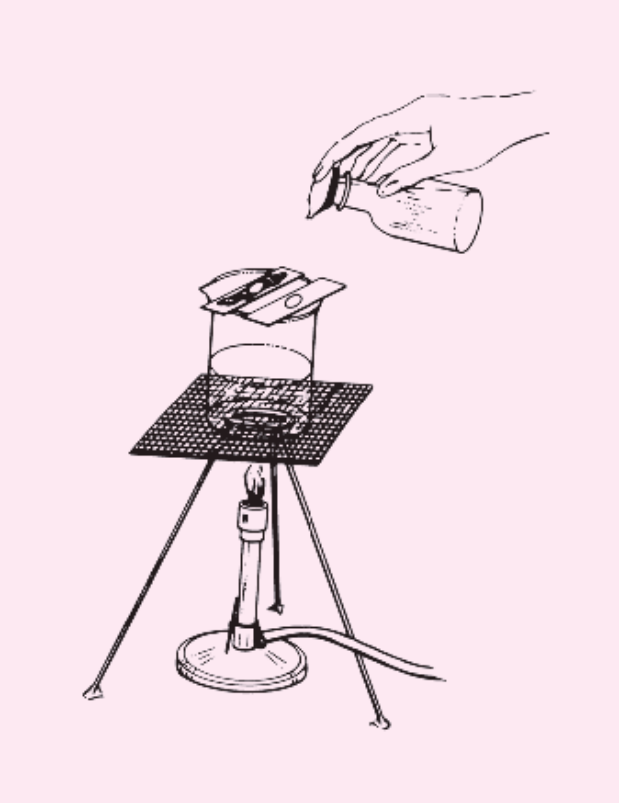


Figure 11 A simple method for applying heat when staining smears, using the endospore stain technique.